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TARGETING PROTEINS TO THE NUCLEAR MEMBRANE: A GENOMIC STUDY IN *SACCHAROMYCES CEREVISIAE* USING Trm1 AS A REPORTER

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ABSTRACT

Appropriate nuclear membrane structure is important for all eukaryotic organisms as evidenced by the numerous human diseases and alterations in gene expression caused by inappropriate targeting of proteins to the inner nuclear membrane (INM). We report here the first genome-wide screen to identify proteins functioning in INM targeting. We transformed to near completion the 4850 members of the Saccharomyces cerevisiae deletion collection of unessential genes in the 96-well format with a plasmid encoding a reporter protein, Trm1-II-GFP, which normally resides at the INM. We found that deletion of genes encoding subunits of the N-terminal acetyltransferase, NatC, cause mislocation of Trm1-II-GFP from the INM to the nucleoplasm. Mass spectroscopic analysis indicates that Trm1-II-GFP is N-acetylated. N-terminal mutations of Trm1-II-GFP predicted to ablate N-acetylation cause nucleoplasmic location, whereas a variant with an N-terminal alteration predicted to allow N-acetylation by NatC is located at the INM, providing genetic support that Trm1p-II N-acetylation is necessary for its subnuclear INM location. However, because N-acetylation appears not to be sufficient for INM targeting, it may provide a necessary role for INM targeting by affecting Trm1-II-GFP structure and exposure of cis-acting INM targeting motifs. We also discovered that YIL090W/Ice2p, an integral membrane protein located in the endoplasmic reticulum, is necessary for efficient targeting of Trm1p-II-GFP to the INM. YIL090W/Ice2p may serve as a tether for INM proteins or as a regulator of INM tethers. Our methodology can be extrapolated to obtain genome-wide perspectives of mechanisms necessary to achieve appropriate subcellular and/or suborganellar location for any resident protein.

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LIST OF ABBREVIATIONS

AMP	-	ampicillin
dATP	-	deoxyadenosine triphosphate
DAPI	-	4', 6'- diamidino-2-phenylindole dihydrochloride
dNTP	-	deoxynucleotides
DSP	-	Dithiobis(succinimidylpropionate)
DTT	-	1, 4-dithiothreitol
EDTA	-	ethelenediaminetetraacetic acid
ER	-	endoplasmic reticulum
G418	-	Geneticin
GDW	-	glass distilled water
GFP	-	green fluorescent protein
dNTP	-	deoxynucleoside triphosphate
INM	-	inner nuclear membrane
Kb	-	kilobase
KAN	-	kanamycin
NPC	-	nuclear pore complex
NLS	-	nuclear localization sequences
NTT	-	Nuclear tether for Trm
ORF	-	open reading frame
ONM	-	outer nuclear membrane

PEG	-	polyethylene glycol
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
RPM	-	revolutions per minute
SDS	-	sodium dodecyl sulfate
SBP	-	spindle pole body
TBE	-	Tris, boric acid, EDTA
TE	-	Tris, EDTA
tRNA	-	transfer RNA
dTTP	-	deoxythymine triphosphate
YEPD	-	yeast extract, peptone, dextrose
YT	-	yeast tryptone

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CHAPTER ONE

GENERAL INTRODUCTION

The major characteristic of a eukaryotic cell is the sub-compartmentalization of the various cellular functions into different organelles (Fig1). All these organelles reside within a mesh of cellular cytoskeleton called cytoplasm. The organelles are typically separated from each other and the cytoplasm by a double layered lipid membrane. The nucleus stores the genetic material and is the functional hub of the cell, the mitochondria carries out the respiratory activities and is the powerhouse, the endoplasmic reticulum(ER) and golgi carry out the protein sorting as well as secretory functions. Though many of these processes are still unclear, they are carried out to perfection within the cell with each of the organelles doing their specific functions. This division of labor within the cell requires the more general processes such as such as protein synthesis, and other chemical reactions to occur in the cytoplasm. Proteins synthesized in the cytoplasm have to be targeted to various organelles where they carry out their specific functions. The targeting machinery recognizes the cellular address on the protein, in the form of specific domains or amino acid motifs, and zips them to the appropriate locations. Previous studies have enabled the recognition of signals for sorting to specific organelles such as the mitochondria, nucleus, plasma membrane etc. However, many questions still remain unanswered. This study is directed towards understanding the targeting of proteins to the inner nuclear membrane (INM), and deciphering the genes that may be involved in this process.

Figure 1: displays the compartmentalization of an eukaryotic cells into organelles, each separated from the cytoplasm and other organelles by a membrane. Reproduced from <u>micro.magnet.fsu.edu</u>



Nuclear Membrane

Each of the organelles is important for proper functioning of the cell. The functional core of the cell is the nucleus. The nucleus encompasses the genetic material within a double layered nuclear membrane (Fig.2). The Outer nuclear membrane (ONM) that excludes the cytoplasm and is continuous with the endoplasmic reticulum (ER) and the Inner nuclear membrane (INM) that surrounds the nucleoplasm. The continuity of this membrane is disrupted at various sites by the nuclear pore complex (NPC). The NPC is a complex of over 30 different proteins and plays an important role in trafficking of molecules into and out of the nucleus (Fig.2). The nuclear membrane harbors many proteins, integral membrane proteins as well as peripheral proteins. Integral membrane proteins may span only through the ONM or the INM or across the double layered envelope. Many theories have been put forth to explain the movement of integral membrane proteins from the cytoplasm to the nuclear membrane, especially the INM. Of these, the most accepted is the 'Diffusion Retention model' (Holmer and Worman, 2001). This model takes advantage of the fact that the ONM is continuous with the ER. According to the model, the proteins that are predestined to the nuclear membrane are pulled into the ER while being synthesized on the ribosomes attached to the ER. These proteins then move via passive diffusion from the ER along the continuity of the ER membrane and the ONM, through the lateral channels and are then integrated into the INM. Another new study proposes this diffusional movement to rely on energy

Figure 2: The picture displays the structural characteristics of the double layered nuclear membrane of a mammalian cell. It displays the presence of nuclear pore complex (NPC), integral membrane proteins and peripheral membrane proteins. Reproduced from

http://www-ermm.cbcu.cam.ac.uk



dependent restructuring of the NPCs that create transient channels at the nuclear envelope (NE) (Ohba et al., 2004). While many ongoing arguments attempt to explain targeting of integral proteins to the INM, the question of how peripheral membrane proteins are targeted specifically to the INM is not well understood. By one model, proteins are synthesized in the cytoplasm; they then move into the nucleus and interact with a membrane tether that retains the peripheral protein to the INM, preventing their diffusion in the nucleoplasm.

In higher eukaryotic cells, the nuclear envelope (NE) structure is stabilized by peripherally associated intermediate filament proteins that form a meshwork below the INM (Fig.2). These proteins, Lamins polymerize to form a filamentous network. The lamins perform numerous functions within the cell; however, one widely acknowledged role is mechanical support for the NE. Vertebrates including humans have three known lamins genes LMNA, LMNB1 and LMNB2 (Taddei *et al.*, 2004) which encode seven different alternatively spliced variants. Less complex organisms have fewer numbers of lamin proteins, for example *C. elegans* has only one lamin gene and *Drosophila melanogaster* has two lamin genes (Taddei *et al.*, 2004). Lamins have not been discovered in plants and unicellular eukaryotes. Homology searches have not revealed the presence of a gene/protein with sufficient similarity in these organisms. However, it is possible that proteins performing a function similar to the lamins, albeit different in sequence, do exist in these simpler organisms.

In mammalian cells, lamin B is the only lamin that is expressed in all cell types. Lamins A and C and its other splice variants are expressed only in differentiated cells and in specific cell types. Lamin B mutant mice are embryonic lethal (Harborth *et al.*, 2001) while mice with mutations in other lamins have been generated and studied as models for various diseases.

Peripheral proteins targeted to the INM are secured at their location via proteinprotein interaction with integral INM proteins, nuclear lamins, and other peripheral INM proteins or via lipid-protein interaction with the membrane. Proteins of the INM perform various important functions. These proteins interact with chromatin and play a role in the transcriptional regulation of genes and gene silencing and establishment of heterochromatin (Hetzer *et al.*, 2005). Thus, targeting these proteins to their location is vital for proper functioning of various cellular processes.

Nuclear envelope and diseases

Over the last few years the number of diseases attributed to mutations in components of the NE has been rapidly increasing. Mutations in these components may affect the mechanical strength of the NE causing it to become fragile, they may affect regulation of gene expression (Worman and Courvalin, 2005) and/or one of the as yet unknown functions performed by the numerous proteins associated with the NM. Disruption of any of these activities may affect proper functioning of the cell and lead to manifestation of diseases.

Most of the diseases discovered to date have been attributed to mutations in the lamins, lamin A, lamin C, lamin B receptor (LBR) and the integral membrane proteins that the lamins interact with such as emerin and Man1. These diseases, resulting due to

mutations in lamin or lamin associated proteins are broadly referred to as laminopathies. LBR is an integral NE protein that was identified by its ability to bind lamin B1. It also binds to heterochromatin protein 1(HP1) and DNA (Hetzer *et al.*, 2005). LBR is homologous to yeast sterol C-14 reductase. It has been shown to have this activity when expressed in yeast (Silve *et al.*, 1998). Mutations in LBR cause Pelger-Huet anomaly and Greenburg skeletal dysplasia (Worman and Courvalin, 2005). One of the most common and well studied diseases is Emery-Dreifuss muscular dystrophy (EDMD). This is caused due to mutation in the integral INM protein emerin. MAN1 has been linked to 3 related bone disorders: osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis. MAN1 and emerin linked diseases affect different tissues in humans, however in *C.elegans*, the homologues of these two genes are synthetically lethal, suggesting that they probably function in the same pathway (Liu *et al.*, 2003a).

The genomic era has steered many large scale studies to unearth genes involved not only in diseases but also in various specific processes, gene expression, transport, targeting of proteins to different sites within the cell, identification of interacting proteins, identification of proteins interacting with DNA, so on and so forth. Simpler model systems like *S.cerevisiae* and *C.elegans* have proved to be valuable for carrying out such genome wide analysis and studies. Despite the obvious differences, the organisms and the simple functioning of the cell is highly conserved and more often than not, answers discovered in these simple model systems provide solutions for questions in the more complex mammalian systems. The genes and gene products involved in targeting the peripheral proteins or tethering of these proteins to the INM have not yet been identified. This was the puzzle we set out to unravel.

Endoplasmic Reticulum (ER)

The ER is the major site for lipid biosynthesis, protein folding, disulphide bond formation, various modifications of proteins such as core glycosylation, and the entry point into the secretory pathway. Based on its functionality the ER can be described as rough endoplasmic reticulum (RER) or smooth endoplasmic reticulum (SER). The ER membrane around the nucleus harbors a large number of ribosomes and is referred to as RER while the tubules towards the cell periphery are devoid of ribosomes and are called SER. Many of the proteins that need to be sorted into different compartments of the cell or are secreted outside the cell are synthesized on the RER from where the nascent peptide chains are transferred into or across the ER membrane as they are being synthesized. These proteins undergo modification within the ER, and are then exported in lipid coated vesicles to other organelles (Mattaj, 2004).

In eukaryotic cells, the endoplasmic reticulum forms a continuous network of tubules and cisternae that is continuous with the ONM and extends to the cell periphery. Based on its location, the ER tubules that are directly connected with the ONM are referred to as perinuclear ER. The ER membrane extends to the cell cortex and pushes against the plasma membrane. This part of the ER network is referred to as the cortical ER. The perinuclear and cortical ER are connected by a few tubules. Although the cortical and the nuclear ER are spatially distinct they have similar functions. The ER tubule network is highly dynamic and continuously rearranged by tubules sliding along one another, new tubules emerging from preexisting one and fusing with others (de Martin *et al.*, 2005). Little is known about how this complex morphology of the ER is formed and maintained or what role the structure plays in function of the ER. It has been thought that microtubules play a role in this process as drugs that cause depolymerization of microtubules cause reversible contraction of ER to the center of the cell. Newly formed tubules are thought to be pulled out of existing tubules towards the cell periphery by motor proteins sliding along microtubules (Powell and Latterich, 2000). However, in vitro formation of ER network has been shown not to depend on microtubules (Dreier and Rapoport, 2000). Though, the studies do suggest the necessity of microtubules in the maintenance of ER structure in mammalian cells. In contrast, microtubules do not coalign with ER in yeast (Terasaki *et al.* 1986). Furthermore, use of microtubule depolymerizing drugs such as nocodazole has shown that microtubules are not required for either maintenance or dynamics of the ER in yeast (Prinz *et al.*, 2000).

Prinz *et al* (2000) also used drugs such as latrunculin A that disrupts actin structures and showed that although the structure of the cortical ER is not affected, it does affect the dynamics of the ER. Some ER tubules seem to align with actin, however most of them do not. This suggests the presence of a hitherto unknown mechanism involving the actin cytoskeleton that functions in maintaining this complex structure. In a screen for genes affecting ER structure, Prinz *et al* (2000) found yeast mutants that affect ER-golgi trafficking and the signal retention pathway (SRP) to play a role in ER structure.

The ER and the ONM are continuous; furthermore, in yeast which undergoes closed mitosis, the perinuclear ER is inherited by its pulling into the growing bud with the NM. Our finding that a gene involved in inheritance of cortical ER plays a role in INM targeting of proteins further suggests that the maintenance as well as inheritance of NM and ER may be linked to some of the proteins functioning in both pathways.

Nuclear division, Spindle pole body and microtubules

In plants, mammalian cells and other higher eukaryotic organisms, cell division is characterized by the break down of the nuclear envelope during interphase, alignment of the condensed chromosomes across the equatorial plane, attachment of the spindle, separation of the chromatids to opposite poles and reformation of the nuclear envelope after chromosome segregation and ending in cytokinesis. Dissolution of the nuclear membrane overcomes the differences in the concentration of soluble proteins that exists across the envelope. This process of cell division is referred to as 'open mitosis'. However, in lower eukaryotes such as the budding yeast, the nuclear envelope remains intact throughout the mitotic process. Such a cell division is referred to as 'closed mitosis'. The NE elongates into the newly formed bud and is then chipped off to form an intact nucleus within the new bud. There is speculation that during closed mitosis, the nuclear envelope may become much more leaky and permeable. A recent report provides evidence for this in Aspergillus niduluns (De souza et al., 2004, Sazer, 2005). However, evidence has been provided for leakiness of the nucleus, in either no

Schizosaccharomyces pombe or *Saccharomyces cerevisiae* since soluble proteins do not seem to equilibrate in either yeast during mitosis (Sazer, 2005).

One of the hallmarks of mitosis is the formation of the spindle filaments. In the organisms with open mitosis, centrioles are formed at both poles and formation of astral filaments called spindle occurs from the centrioles. In yeast, closed mitosis and the presence of NE entails the formation of intranuclear spindle from the spindle pole body (SPB). The SPB is the sole site of microtubule organization in yeast (Jaspersen and Winey, 2004). SPBs are embedded in the NE throughout the life cycle of yeast and are the site of nucleation for both nuclear as well as cytoplasmic microtubules. It is a multiprotein structure that is viewed as a single darkly staining spot on the NE. The SPB is a cylindrical organelle that consists of three disks or plaques. The outer plaque faces the nucleoplasm and is associated with nuclear microtubules and the central plaque spans the nuclear membrane.

One of the major functions of SBP is to nucleate microtubules from α/β tubulin dimers. This provides polarity to the microtubules such that the minus ends are at the SBP and the plus ends extend into the cytoplasm and nucleoplasm. Microtubule polarity is important for directional movement of cargo along the microtubule by plus or minus end directed motor proteins. γ -tubulin is a conserved component required for nucleation, while the microtubule filament itself is composed of α and β -tubulins. In yeast γ -tubulin is encoded by *TUB4*, and α and β tubulins are encoded by *TUB1* and *TUB2* respectively. The SPB is responsible for two classes of microtubules, nuclear and cytoplasmic. Cytoplasmic microtubules are used for karyogamy while nuclear microtubules function in nuclear positioning and spindle orientation (Winsor and Schiebel, 1997).

To date only 17 components of the multiprotein SPB have been identified. Many of these are essential and deletion of any of the SPB components results in gross defects in SPB structure and function as well as in microtubule formations which, in turn, have various secondary defects. One of the essential components of SPB is a coiled-coil protein Spc42. This forms the central core of SPB. Florescent tagged Spc42 can be observed as a single small spot on the NE. Spc42 along with Spc110 and Spc29 have been shown to interact with an INM peripheral protein Mlp2 (Niebel *et al.*, 2005) and thereby facilitate the insertion of SPB in the INM. The functions performed by the Mlp proteins (Mlp1 and Mlp2) have not been clearly defined though they have been implicated in nuclear retention of unspliced mRNA.

Trm1-II-GFP has often been observed to form single bright spots on the NM indicating accumulation of the protein at one spot on the NM. This finding led us to suspect that the nuclear rim localization of Trm1-II may be achieved by a two stem process step 1) accumulation at a single spot and spread around the NM. SPB components localize in a manner similar to Trm1-II-GFP accumulation, therefore they might facilitate the initial contact of Trm1-II to the NM and aid in its spread around the membrane

Modification of proteins

Proteins may be modified in a co-translational or post-translational manner. Proteins are modified in many ways and each of these modifications seems to play specific roles in the functionality of the protein. Some of the common modifications occurring within the cell include proteolysis, phosphorylation, methylation, acetylation and glycosylation. The functions of many modifications are still unknown (Clark *et al.*, 2005).

Many proteins are synthesized in an inactive precursor form, for example trypsin, insulin, chymotrypsin etc. These proteins are cleaved at the N-terminus to activate the enzyme. Methylation of histories attached to the chromatin aids in silencing the region of the chromatin, while acetylation of the same protein, functions to open up the chromatin for active transcription (Clark et al., 2005). Phosphorylation is a well known and well characterized form of activating or deactivating proteins in signal transduction pathways. This enables the cell to interpret extracellular condition into cell regulatory mechanisms as in case of activation of signal transduction pathways in response to signals such as nutritional excess, nutritional starvation, presence of chemicals, temperature stress, presence of pheromones and various other processes. All these environmental signals trigger a chain of reactions within the cell, a sensor on the cell surface recognizes the signal and conveys it within, allowing the cell to respond in an appropriate manner. Glycosylation is most commonly found on membrane proteins. It involves the addition of complex carbohydrates to the amide or hydroxyl group of the proteins and is carried out within the ER.

This thesis describes the identification of a novel role for acetylation in protein targeting. Acetylation may be internal, i.e. the amino acids within the protein are acetylated (mostly lysines) or it may be at the N-terminus of the protein. The modification of proteins plays a role in their interaction with other proteins or with cellular components such as a lipid membrane. Recently, these modification based interactions have been shown to be utilized by cellular machinery for sorting the proteins to their respective destination. Here, we report one such novel function for the N-terminal acetylation of the INM protein Trm1.

Yeast as a model system

Yeast is a unicellular model system. Though very different from the multicellular invertebrate or vertebrate model systems, various discoveries have time and again proved the conservation of important processes from yeast to mammals. Many of the important processes were first discovered and studied in yeast such as cell cycle (the basis for many cancer studies), nuclear transport process including the Ran cycle, silencing mechanism and so on. All these processes and more have after initial studies in yeast have been found to be conserved in higher organisms. Being a simple system and well characterized, adds to the list of advantages for using yeast to study targeting of proteins to the INM. We believe that this study will lead to better understanding of how peripheral proteins are targeted specifically to the INM. Such findings can be applied for a better understanding of peripheral INM proteins trafficking in higher eukaryotes and lead to elucidation of the defects in various diseases associated anomalies in the NM. Furthermore, the various genomic collections aid us in employing such studies as are yet not possible in other model systems. The yeast deletion collection is an arrayed collection of over 4000 strains each carrying a deletion of one specific gene which has been replaced by a KANMX marker rendering it resistant to G418 (Winzeler *et al.*, 1999). The Phizicky collection, an arrayed collection over 6000 strains each carrying a plasmid encoding a GST tagged version of one of the proteins encoded in the yeast genome (Martzen *et al.*, 1999). The yeast GFP collection is again a genomic collection where every gene in yeast genome has been chromosomally tagged with GFP enabling the localization of the proteins encoded by these genes (Huh *et al.*, 2003). All these collections aid in analyzing the entire genome for various processes of interest. In this thesis we report the screening of 2/3rds of the yeast genome to identify genes involved in targeting proteins to the INM.

<u>Trm1</u>

tRNAs play a central role in protein synthesis by forming a bridge between mRNA and amino acids. They read the mRNA via their anticodon loop and aid in the addition of amino acid to the extending peptide chain. The tRNAs are modified at various sites by methylation, pseudouridylation, adenosine deamination and so forth. Each of these modifications is carried out by a separate set of enzymes. Of these, methylation is carried out by a group of enzymes called Trm (tRNA methyltransferase). These include at least more than 10 enzymes, each methylating a specific base or bases on the tRNA. Trm1 was identified for its ability to dimethylate tRNA at position 26 (Hopper *et al.*,

1982; Ellis *et al.*, 1986). The exact role of this particular modification is unknown but it may contribute to the stability of the tRNA. Mutations in a serine tRNA have been reported to be synthetically lethal with $trm1\Delta$. Lack of the dimethyl group at G26 causes the mutant tRNA to be unstable in $trm1\Delta$ background (Johansson and Bystrom, 2002). Synthetic lethal effects have also been reported for other tRNA modification genes; for example, $trm11\Delta$ trm1 Δ double mutants are not viable (Purushotaman *et al.*, 2005). Lack of a single modification does not seem to affect the stability of the tRNA molecule however, when tRNA lacks two or more modifications, certain combinations seem to be lethal. Modifications of tRNA have many roles that have only recently come to light. These functions include stabilization of the RNA molecule, maintenance of RNA structure, and base pairing.

Trm1 belongs to a group of enzymes referred to as sorting isozymes (Li et al., 1989). This term signifies a group of more than one protein encoded by the same gene and targeted to different sub-compartments within the cell. The *TRM1* gene codes for two proteins Trm1-I (long form) and Trm1-II (short form) that differ by a 17 amino acid extension at the N-terminus of the long form of the enzyme (Fig.3). The two proteins differ in the site of transcriptional initiation of the mRNA. Trm1-I is exclusively targeted to the mitochondria while Trm1-II is targeted both to the nucleus and mitochondria. 90% of the synthesized Trm1-II is targeted to the nucleus while 10% is localized in the mitochondria (Rose *et al.*, 1992). These two proteins are sorted to different organelles and the information to achieve this resides within the sequence of the protein.

The proteins targeted to the mitochondria have an ampiphatic motif at or in the vicinity of the N-terminus. This motif serves to target proteins to the mitochondria and is referred to as mitochondrial targeting sequence (MTS). Similarly, proteins targeted to the nucleus harbor motifs rich in basic amino acids called the nuclear localization signal (NLS); proteins targeted to the peroxisomes carry peroxisome targeting sequences (PTS) which generally resides at the N-terminus. In the case of sorting isozymes, the same protein tends to carry both signals. In such cases, differential distribution may achieved by (1) Controlling accessibility to the targeting signals by differential folding allowing the exposure of only one of the signals, (2) other proteins may bind to one of the signals and thereby prevent its accessibility to the protein sorting machinery, and (3) posttranslational modification. In case of Trm1, the MTS resides within amino acids 17-48 (Trm1-II) and a NLS is present from amino acids 95-102 (Rose et al., 1992). The MTS by itself is not efficient for mitochondrial sorting as the NLS is the more predominant signal in Trm1-II. The amino acids 1-17 in Trm1-I increase the efficiency of mitochondrial import since the long form localizes exclusively to the mitochondria (Ellis et al., 1987)

Figure 3: The two isozymes of Trm1, Trm1-I with the N-terminal extension localizes to the INM, and Trm1-II localizes largely to the INM. The presence of MTS (1-17) at the N-terminus and the NLS from amino acid 95-102 is also depicted in the figure.



TRM1 genes are found in eukaryotes and archaea bacteria but are absent in eubacteria. The *TRM1* gene in other organisms like humans and mice contain two ATGs within the first 30-40 codons, indicating that these may code for isozymes (Rose *et al.*, 1992).

The N-terminus of human Trm1 when analyzed by helical wheel predicts to have amphiphilic structure characteristic of MTSs. Thus, this protein could be sorted to the mitochondria also. Other sorting isozymes encoded by yeast include, Mod5 sorted to the mitochondria, nucleus and also present in the cytoplasm (Boguta *et al.*, 1994), Cca1 sorted to the nucleus and mitochondria (Wolfe *et al.*, 1994, Wolfe *et al.*, 1996) and Hts1 (histidine tRNA synthetase) translocated to mitochondria, chloroplast and nucleus (Chiu *et al.*, 1992).

The majority of the proteins encoded by the nuclear genome are synthesized within the cytoplasm. The compartmentalization of a eukaryotic cell entails the existence of a well evolved system for sorting these proteins to their respective sub compartments. This would mean that these proteins carry some signal recognized by the cellular machinery allowing it to target the proteins to the different organelles. All the above mentioned sorting isozymes are also encoded by the prokaryotes where it is not necessary to target proteins to specific sub-cellular locations. Thus, they do not require the signal sequences for protein targeting. Based on this assumption, Stanford *et al* (2000) analyzed the protein sequences of many isozymes and found that there exist many domains that are present in eukaryotes but are absent in their prokaryotic counterparts. Further analysis

Figure 4: Schematic of Trm1 protein sequence alignment from mammals, fungi and bacteria. The colored boxes represent the sequences conserved across eukaryotic and prokaryotic species. Black lines represent eukaryotic sequences not generally similar to each other (Stanford *et al.*, 2005)



revealed that the mitochondrial targeting sequence at the N-terminus of the above described proteins were present in the eukaryotic homologs but absent in the prokaryotic proteins. Also, the NLS containing domains were present in all eukaryotic proteins but absent in the prokaryotic homologs. The additional information in the eukaryotic proteins probably serves to direct these proteins to the appropriate subcellular localization. These domains are aptly named ADEPTs (Additional Domains for Eukaryotic Protein Targeting). In the proteins studied to date such as Trm1, Mod5, Rsp5 etc, some of the ADEPTs encompass well characterized motifs like NLS or MTS. However, many of these motifs also comprise of sequences whose functions are unknown. These sequences may provide information important for cellular distribution of eukaryotic proteins and thereby facilitate characterization of novel protein targeting motifs. The sequence of Trm1 revealed the presence of 4 ADEPTs (Fig.4). A parallel study in the lab analyzed the importance of each ADEPT in targeting Trm1 to the INM (Stauffer *et al.*, in prep).

Many organelles have sub-locations for different functions performed by them. Based on its function proteins tend to be targeted to these sub-organeller spaces. For example, within the nucleus proteins may be targeted to SPB, INM, nucleolus, cajal bodies, speckles, gems etc. This requires that these proteins carry multiple signals, one for sorting into the specific organelles and others for the sub-organellar location.

Trm1-II localizes to the INM and forms a ring around the NM. Despite the lack of electron microscopy data, we can confidently state that Trm1-II localizes to the INM because mutations that prevent the tethering of the protein to the INM causes nucleoplasmic accumulation of the protein (Murthi and Hopper, 2005). If Trm1-II was
bound to the ONM, these mutations would result in cytoplasmic accumulation. Furthermore, we know that the methylation of tRNA occurs before they exit the nucleus (Edqvist *et al.*, 1995) and mutation of the nuclear localization signal (NLS) of Trm1-II causes it to accumulate within the cytoplasm (Rose *et al.*, 1992). Trm1-II is tethered to the INM via protein-protein interaction or protein-lipid interaction. Trm1-II-GFP localization is identical to that of the wild type protein. The added advantage of using the GFP fused protein is visualization of protein localization in live cells. The unique localization as well as easy visualization of this protein provided us with an ideal candidate for a reporter to screen for genes involved in targeting proteins to the INM.

This study unearthed a novel function for a modification, N-terminal acetylation, as well as revealed the importance of cortical ER in targeting proteins to subcellular compartments. These results are discussed in detail in the following chapters.

CHAPTER TWO

MATERIALS AND METHODS

Yeast strains and methods

Yeast strains BY4741 (*MATa* his3 Δ leu2 Δ met15 Δ ura3 Δ) and BY4742 (*MATa* his3 Δ leu2 Δ lys2 Δ ura3 Δ) are parents of the deletion collections (Winzeler et al., 1999). The yeast collection with GST-ORF containing plasmids EJ 758 (*MATa* his3-delta200, leu2-3,112, ura3-52, pep4::HIS3) were obtained from E. Phizicky (Martzen et al., 1999). Yeast strains were maintained on YEPD media with or without G418 (0.2 mg/ml) or synthetic defined media lacking appropriate nutritional ingredients. E. coli DH5 α was used for propagation of recombinant DNA plasmids and was maintained in YT media with appropriate antibiotics.

Plasmids

Most PCR reactions were carried out using *Pfu* DNA polymerase (Stratagene). DNAs were ligated with T4 DNA ligase (New England Biolabs). The PSU Coll. Medicine Macromolecular Core Facility generated all oligonucleotides. All constructs were sequenced by the same facility. <u>pRS415-TRM1-II-GFP</u> is a derivative of pGT554 (Li *et al.*, 1989) in the pRS415 plasmid backbone (Sikorski and Hieter, 1989). It encodes Trm1-II-GFP under the control of its endogenous regulatory sequences (details of the construction available upon request). <u>pTRM1-II-GST</u>: pGP54a is pRS416 containing a *GAL1-GAL10* regulatory region (gift from J. Hopper, PSU Coll. Med.). The sequence encoding *S. japonicum* GST was amplified by PCR using pYEX-GST (Martzen *et al.*, 1999) as the template and inserted into pGP54a digested with *SacI* and *XhoI* to generate

pGP54aGST. The *TRM1* ORF beginning at the 2^{nd} AUG was amplified from genomic DNA (BY4741) with oligonucleotides introducing *EcoRI* and *Hind*III sites. The PCR product was cloned into the pTOPO-BluntII-TOPO cloning vector (Stratagene). *EcoRI* and *Hind*III were employed to transfer *TRM1* into pGP54aGST. <u>pGEMT-A1-4</u> and <u>pGEMTA1-6</u> were generated to facilitate mutagenesis by subcloning a 1.4 kb *TRM1* fragment spanned by 2 *Spe*1 sites into pGEMT (Promega) using Taq (Promega). The plasmids were utilized as templates for reverse PCR amplification and site-directed mutagenesis described below and then the mutant regions were exchanged with the corresponding fragment of pRS415-TRM1-II-GFP.

<u>pRS415-TRM1-II-AL₂-GFP</u> contains a deletion of the *TRM1-II* second codon. It was generated by reverse PCR using pGEMT-A1-4 as the template. <u>pRS415-TRM1-II-K₃ \rightarrow E₃-GFP and <u>pRS415-TRM1-II-L₂ \rightarrow F₂</u> were obtained using pGEMTA1-6 as the template. The mutations were introduced employing the QuikChange II kit (Stratagene). <u>pRS415-TRM1-II-(NatC)-GFP</u> encodes Trm1p-II with an ectopic NatC modification sequence. It was generated by reverse PCR using pGEMT-TRM1-II- Δ L₂ as the template, introducing 4 codons (underlined) of the *PUP2* ORF N-terminus at codon 2, encoding the sequence M<u>FLTRK</u>. <u>pRS415-TRM1-II-(NatA)-GFP</u> encodes Trm1p-II with an Nterminal ectopic NatA modification sequence. It was generated by reverse PCR using pGEMT-TRM1-II Δ L₂ as the template introducing 4 N-terminal codons from *RPS0A/YST1*, a known NatA substrate, at codon 2, generating the sequence M<u>SLPA</u>K.</u> <u>YIL090W-GST</u>-YIL090W was amplified by PCR using BY4741 genomic DNA as template and oligonucleotide primers with flanking *Xma*I sites. The product was cloned into pCR-BluntII-TOPO (Invitrogen) to obtain pTOPO-YIL. The YIL090W ORF was transferred from pTOPO-YIL to pGP-54aGST using *Xma*I to obtain an in-frame fusion with GST.

DNA manipulations

Products of DNA restriction enzymes or PCR were resolved on agarose gels of appropriate percentage in 1X Tris borate EDTA (TBE). Gel slices were excised containing the desired digestion fragment or PCR product and purified using the Qiagen gel purification kit according to manufacturer's instructions (Qiagen Science, Maryland). Dephosphorylation of DNA restriction enzyme fragments was carried out by incubation with shrimp alkaline phosphatase (MBI Fermentas) at 37°C for ~1hour and deactivation of the enzyme at 65°C for 20min.

Oligonucleotides

The PSU Coll. Medicine Macromolecular Core Facility generated all oligonucleotides.

- 1. Athula1 : GGC CGC TCT AGA ACT AGT G
- 2. Athula2 : CCT GAA GAA ACC TTC CAT GGC GCC ATC TAC ATT GTA AG
- 3. Athula3 : GC GCC ATG GAA GGT TTC TTC AG
- 4. Athula4 : CGA CAA AAG CGG TTC CAC AG
- 5. Gfpnar1f: GGC GCC ATG TCT AAA GGT

- 6. Gfpnar2R: GGC GCC TTT GTA CAA TTC ATC CAT ACC
- 7. Athula5 : GGC GCC ATG TTG AAG GCT GCT ATA TCC
- 8. Athula6 : GGA TAT AGC AGC CTT CAA CAT GGC GCC TCC GTG TAA ATT TGC
- 9. Athula7: GGC AAG TCT AGA GGT CCG GCT G
- 10. Athula8: CTC AAG ATC TAG TCA AAA GTG CAT
- 11. Athula9: TGA TAG TTA TCA TGT TTC TTA TCC
- 12. Athula10: ATT AGC AGC GCT AGG AGC TTC
- 13. Athula23: AAG CTT ATG ACC AAG TTA CCT ATA CTA GT TAT TGG
- 14. Athula24: CTC CAG TGA TCC GAT TTT GGA GGA TGG TCG CCA C
- 15. Athula27: GCA AAT TTA CAC GGA ATG TTG GAG GCT GCT ATA TCC AAA

ATT AA

- 16. Athula29: AAG CTT CAT TTC AAC ATC CGC TTC TTC TGG
- 17. Athula32: GTC GAC ATG ACC AAG TTA CCT ATA CTA GGT TAT TGG
- 18. Athula34: GG GCA AAT TTA CAC GGA ATG GAG AAG GCT GCT ATA TCC AAA A
- 19. Athula35: T TTT GGA TAT AGC AGC CTT CTC CAT TCC GTG TAA ATT TGC CC
- 20. Athula36: G GCA AAT TTA CAC GGA ATG TTT AAG GCCT GCT ATA TCC AAA ATT AAA
- 21. Athula37: TT TAA TTT TGG ATA TAG CAG CCT TAA ACA TTC CGT GTA AAT TTG CC

22. Athula38: GAA TTC ATG TTG AAG GCT ATA TCC 23. Athula40: AAG CTT TGA AGT GTT GGG ACG GGC TTT TGG 24. Athula41: CCC GGG ATG ACC AGT TTG TCC AAA AGC TTC ATG 25. Athula42: CCC GGG ACT ACC AGA ACC TAT TAA TTC TGT AGC

Chemical transformation

DH5 α *E. coli* cells were thawed on ice. 2.5 µl mini-prep DNA (Qiagen miniprep. Kit, Qiagen) was added to 200ul chemically *E. coli* competent cells and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 90 sec and then 250 µl LB was added. The cells were grown at 37°C for 1 hr and then plated on YT media containing 50 µg/ml ampicillin or 50 µg/ml kanamycin. The plates were then incubated at 37°C overnight and resulting single colonies were isolated.

Preparation of electrocompetent E. coli cells

A 5 ml culture of DH5a cells was grown overnight at 37°C in LB with no selection. The 5ml culture was used to inoculate a 500 ml culture of LB with no selection. This culture was grown for ~4hr at 37°C with shaking until an OD of 0.600 was reached. Cells were then transferred to 250 ml centrifuge bottles and chilled on ice for ~20min. These cultures were pelleted and washed with cell wash 1M HEPES solution. Cell pellets were resuspended in and frozen in 80 μ l aliquots at -80° C.

Electroporation

DH5 α *E. coli* cells were thawed on ice. 2.5 µl mini-prep DNA was added to 80 µl electrocompetent cells. Cells were transferred to chilled Gene Pulser cuvettes and electroporated using the settings: 2.5 KV, 25 µF and 2000hms. 250 µl YT was added to the cells and the cultures were transferred to microfuge tubes. Cultures were grown for 45 mins at 37°C shaking horizontally. Cultures were then plated on YT media containing 50 µg/ml ampicillin and incubated overnight at 37°C.

Isolation of plasmid DNA from E. coli

Plasmid DNA was obtained using the Qiagen mini-prep kit following manufacturer's instructions (Qiagen Sciences).

One-step yeast transformation

250 μl yeast cultures were pelleted for each yeast transformation. 2.5 μl mini-prep DNA was added along with 100 μl 1-step buffer (0.1M DTT, 100μg sssDNA, 0.2M LiAc, 40% PEG 3350). Cultures were incubated at 42°C for 30 mins and then plated on appropriate media. Cultures were grown for 2-3 days at 23 or 30°C.

96-well transformation

The procedure is based on the LiAc transformation protocol (Ito *et al.*, 1983). Strains were grown in YEPD + G418 in 96-well plates at 23° C for 2 days, and then collected employing a Jouan CR412 centrifuge with an adapter accommodating 96-well plates.

The media was aspirated and cells were resuspended in 100 µl transformation mix (41% PEG, 0.2M LiAc, 0.1M DDT, 0.5 µg boiled single stranded sheared salmon sperm DNA) and 1 µg plasmid DNA. After heat shock at 45°C for 45-60 min, transformants were selected by transferring aliquots to new plates containing selection media and grown at 23° C for 3-4 days. By this method ~90% of the wells contain transformed cells.

Site directed mutagenesis

QuikChange site directed mutagenesis kit (Stratagene) was used. Mutagenesis was carried out as per the manufacturer's instructions

Indirect immunofluorescence

The indirect immunofluorescence procedure was carried out as described by Pringle *et al.* (1991) with the modifications previously described by Hopper *et al.* (1990). 10 ml overnight cultures of yeast cells were grown to mid-log phase. 1.2 ml of 37% formaldehyde solution (Fisher Scientific) was added to the culture prior to collection. Cells were collected in a tabletop centrifuge at ~2,000 x g for 5 min at room temperature. Cells were washed once with 5 ml of Solution A (40 mM K₂HPO₄/KH₂PO₄, 500 μ M MgCl₂) then resuspended in 5 ml Solution A with 0.6 ml of 37% formaldehyde. Samples were incubated at room temperature for 1-1.5 hr to fix the cells, centrifuged at ~2,000 x g for 5 min, and then washed twice with Solution B (40 mM K₂HPO₄/KH₂PO₄, 500 μ M MgCl₂, 1.2 M sorbitol). Cells were resuspended in 250 ul Solution B, 13.75 μ l glusulase (NEN), 2.5 ul β-mercaptoethanol (Sigma), 5 μ l zymolyase 20T (1 mg/200 μ H₂O) (ICN Biomedicals, Inc.) and incubated at 37°C for 20-30 min. Samples were centrifuged at ~2,000 x g for 5 min., washed once with Solution B and resuspended in Solution B. 10 μ l of cell suspension was loaded into each well of a glass slide coated with 1:10 poly-Llysine (Sigma), and liquid was aspirated. Each well was blocked with 10 μ l Solution F (7.4 X 10⁻⁴ M KH₂PO₄, 0.15 M NaCl, 0.015 M NaN₃, 0.1%BSA) for 1 hr, after which the solution was removed. 10 μ l of primary antibody diluted in Solution F was applied to each well, incubated for 1 hr at room temperature and removed. Wells were washed five times with Solution F. The incubation and washing steps were repeated with secondary antibody diluted in Solution F. A 100 ng/ml solution of 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) in H₂O was prepared and 20 ul of it was placed in each well for 1 min before its removal. Wells were washed once with H₂O. Mounting media (5.5 mM phenylene diamine dihydrochloride, pH 9.0; 90% glycerol) was added to the slide prior to fastening a cover slip on top with nail polish.

Processing of fluorescent images

Fluorescence images were obtained using a Nikon Microphot-FX microscope equipped with a 60x objective and a SenSys CCD camera (Photometrics Ltd, Tucson, AZ). Image processing was done using QED software (Pittsburgh, PA) and Adobe Photoshop.

Microscopic imaging

Images were obtained using a Nikon Microphot-GX microscope equipped with a 60x objective and a SenSys CCD camera (Photometrics Ltd, Tucson, AZ). Image processing was performed using QED software (Pittsburgh, PA) and Adobe photoshop.

Western Blot

The proteins were separated by electrophoresis using NuPAGE bis-tris gels. The gel was transferred on to a PVC (PolyVinyl Chloride) membrane by electroblotting. The non-specific binding sites were blocked by incubating the membrane in 5% non-fat milk in PBS-T (Sodium hydrogen phosphate, Disodium hydrogen phosphate, Sodium chloride, Tween 20) for 1hr. The excess blocking agent was washed off with PBS-T twice for 5min each. Appropriate concentration of the primary antibody was diluted in 1%BSA/PBS-T and incubated for 1hr. The unbound antibody was removed by washing with PBS-T 4 times for 5min each. The required concentration of HRP-labeled secondary antibody was diluted in 1% BSA/PBS-T and incubated with the membrane for 1hr. The unbound antibody was removed by washing with PBS-T as before. For protein visualization, the chemiluminescence reagent was prepared by mixing equal volumes of Enhanced Luminol reagent and Oxidizing reagent (Pierce). The membrane was incubated in this mix for 1min. The excess reagent removed by blotting and proteins were detected by exposing the blot to Kodak biomax light film.

Genomic DNA from yeast

Cells were grown in an appropriate media to saturation. 1.5ml of the culture was pelleted and resuspended in 300 solution A (0.9M sorbitol, 0.1M EDTA pH 7.5, 150 μ g/ml zymolyase and 14 mM beta-mercaptoethanol) and incubated at 37oC for about 1hr to digest the cells. After checking for spheroplast formation, they were pelleted and resuspended in 300 ul solution B (1% SDS in TE) in order to lyse the cells. 100 ul of solution C was added to this mix in order to precipitate the protein (5M ammonium acetate pH 7).

Plasmid rescue from yeast

Yeast strains were grown to saturation in selection media. 1.5ml of the cells were harvested and resuspended in 200 ul yeast mini-prep mix (1% SDS, 100mM NaCl, 2% tritonX-100, pН 10mM Tris-HCl 8.0, 1mM EDTA). 200ul of phenol:chloroform:isomylalcohol (25:24:1) was added to the resuspend cells. 0.3 g acid washed glass beads were added to this and vortexed for 2min to break open the cells. The mix was centrifuged for 5min, 9000rpm. The aqueous phase was transferred to a fresh tube. The DNA was precipitated out with ethanol and MgCl2. The precipitated DNA was then used to transform into bacteria by electroporation.

DNA sequencing

DNA sequencing was carried out by the Core Facility at the Pennsylvania State University College of Medicine.

Protein Purification

Trm1-II-GST was purified from BY4741 as published (Phizicky *et al.*, 2002). Protein was eluted from Glutathione Sepharose-4B resin (Amersham Biosciences) in 3 ml extraction buffer and concentrated to 0.4 μ g/ul using a Millipore centrifugal filter device (50K). Protein concentration was determined by the Coomassie assay (Pierce). Protein purity was assessed using the NuPage 10% gels tricine gels (Invitrogen) followed by coomassie blue or silver staining. Protein identity was verified by Western analysis employing monoclonal anti-GST (B-14, Santa Cruz) as primary antibody.

Mass spectrometry

Analyses were performed in the Mass Spectrometry/Proteomics Core Facility at the Pennsylvania State College of Medicine. 2 µg of Trm1-II-GST protein was digested with Glu-C endoproteinase (Roche) with a protease to sample ratio of 1/100 (w/w). Digestions were carried out in 50 mM NH₄H₂PO₄ (pH7.8) with 10% (v/v) acetonitrile (AcN) for 10-15 hr at 25°C. Samples were separated on an Eksigent NanoflowLC separation system using a Microm Magic C18 column 5 micron 0.1 X 150 mm nanoflow column in 2% AcN 0.1% trifluoroacetic acid (TFA), and then eluted with a linear gradient over 34 min from 2% AcN, 0.1% TFA to 90% AcN, 0.1% TFA at a flow rate of 90 nl/min. Alpha-cyanohydroxycinnamic acid (5 mg/ml, 70%CH₃OH, 0.1% TFA) was used as the MALDI matrix and loaded into the syringe on an LC Packings-Dionex ProBot, and was added to the sample eluate through a post-column T-junction. The ProBot deposited samples (300 nl sample eluate, 500 nl matrix solution) every 20 s onto

Applied Biosystems MALDI target plates. After drying the plates were placed in the autoloader of an Applied Biosystems 4700 Proteomics Analyzer MALDI TOF-TOF, and 1000 laser shots per sample spot were automatically collected from 40 randomly chosen spots within each sample spot. Before sample spectra were taken masses were calibrated for each sample plate using 6 spots containing 5 known calibrants.

In vivo Crosslinking

Cells were grown in 5ml cultures and used to seed 100 ml culture which in turn was used to inoculate 1 Lt culture. The cells were grown to O.D ~0.7-1.0. Cells were collected by centrifugation. The cell pellet was resuspended and incubated in 100 mM Tris pH 9.4/10mM DTT for 10 min at room temperature. The cells were then washed with XL buffer (20 mM Sodium Phosphate pH 7.5, 150 mM Sodium chloride, 1M sorbitol) and resuspended in 100 ml XL buffer containing 125 mM PMSF. These cells were then divided into 2 falcon tubes. 100 ul of the crosslinker DSP (20 mg/ml in DMSO stock) was added into one of the tubes while DMSO was added in the other falcon tube. 100 ul zymolyase (5 mg/ml stock in XL buffer) was added to both the tubes in order to generate spheroplasts. The tubes were incubated while shaking at 30°C for 25min. From then onwards, the procedure was carried out in the cold room at 4°C. After digestion, the spheroplasts were pelleted and resuspended in XL buffer. The cell pellet was then solubilized in 5ml SUME buffer (1% SDS, 8 M urea, 10 M MOPS buffer pH 6.8, 5 mM EDTA, 1% NP-40, 0.5% Sodium deoxycholic acid) containing protease inhibitors and

200 µM hydroxylamine. The solubilized cells were transferred into eppendorf tubes and vortexed in the cold room to lyse the pellet. This suspension of lysed cells was incubated while rotating for 10 min and centrifuged at 10,000 rpm for 20 min. The supernatant was transferred into new falcon tubes containing 5 ml HNTG buffer (20 mM HEPES, pH 7.5, 300mM NaCl, 1% Triton X-100, 10% glycerol). This cell lysate was loaded onto IgG sepharose column (Ambersham Pharmacia). The column was washed and protein eluted as per the manufacturer's protocol.

Protein Gels

All protein gels used in this study were 10% NuPAGE Novex Bis-tris gels (Invitrogen).

Silver staining

Silver staining of Trm1-GST purification gels were carried out using BioRad silver stain plus kit. The silver staining of all cross linking gels were carried out by the following procedure. The gels were first fixed in fixing solution (40% absolute ethanol, 10% glacial acetic acid) for about 1-2 hrs. The gel was then transferred to sensitizing solution (30% v/v ethanol, 6.8% w/v sodium acetate, 0.2% w/v sodium thiosulphate, 0.125% v/v glutaraldehyde) for 30 min. After washing with distilled water 3 times for 5min each, the gel was placed in silver solution (0.25% w/v silver nitrate, 0.015% v/v formaldehyde) for 20min. Silver nitrate was washed off with distilled water. The gel was then developed in developing solution (2.5% w/v sodium carbonate, 0.0074% v/v

formaldehyde). Once the protein bands were clearly visibly, the reaction was stopped by replacing the developing solution with stop solution (1.5% w/v EDTA disodium salt) for 30 min. Finally the gel was stored in preserving solution (30% v/v ethanol, 4% v/v glycerol).

InGel digestion

In gel Trypsin digestion was carried out using InGelTM kit from GenoTech. The protocol was followed as per the manufacturer's instructions.

CHAPTER 3

ABSTRACT

Appropriate nuclear membrane structure is important for all eukaryotic organisms as evidenced by the numerous human diseases and alterations in gene expression caused by inappropriate targeting of proteins to the inner nuclear membrane (INM). We report here the first genome-wide screen to identify proteins functioning in INM targeting. We transformed to near completion the 4850 members of the *Saccharomyces cerevisiae* deletion collection of unessential genes. The transformation was carried out in the 96-well format. The transformed plasmid encoded a reporter protein, Trm1-II-GFP, which normally resides at the INM. We found that deletion of genes encoding subunits of the N-terminal acetyltransferase, NatC, cause mislocation of Trm1-II-GFP from the INM to the nucleoplasm. We also discovered that YIL090W/Ice2, an integral membrane protein located in the endoplasmic reticulum, is necessary for efficient targeting of Trm1-II-GFP to the INM. YIL090W/Ice2 may serve as a regulator of INM tethers. Our methodology can be extrapolated to obtain genome-wide perspectives of mechanisms necessary to achieve appropriate subcellular and/or suborganellar location for any resident protein.

INTRODUCTION

Studies in yeast have during the past few years shifted towards large scale analyses involving the entire genome. Yeast has a total of over 6000 genes of which 2/3rds or 4700 are non-essential and the rest ~ 1500 are essential and are required for the viability of the strains. The comparatively small genome size makes yeast an ideal candidate for use in screening the entire genome for various processes. This has led to the development of various genome wide collections to aid in such large scale studies. The yeast deletion collection (Winzeler et al., 1999) was the first such collection to be developed. Since the study described here was based on that collection, it is the only collection that will be described in detail.

Yeast Deletion Collection

The yeast deletion collection was developed by a large consortium of American and European laboratories. Each non-essential ORF in yeast was deleted and replaced by a G418 resistance marker and arrayed in a collection of over 4700 strains, each containing a single gene deletion. The PCR based deletion strategy used to create this collection is depicted in Fig.5. The primers used in the first PCR reaction were about 74 bp long. These were designed to have 18 bp genomic sequence that flank either 5' or 3' end of the ORF (directly proximal and distal to the start and stop codons respectively), a 20 bp unique sequence (the 'molecular bar code' TAG), an 18 bp common to all gene

Figure 5: PCR strategy for construction of the deletion collection. First round of PCR used primers with molecular bar code to amplify the KANMX gene. Second round of PCR used primers with regions homologous to the start and end of the gene to be deleted to amplify KANMX and this PCR product was transformed into wild type yeast strain to allow homologous recombination to delete the gene of interest



Chromosomal integration by homologous recombination

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http://wwwsequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html

disruptions, and finally an 18 bp sequence homologous to KANMX4 cassette. In a second PCR reaction, the above product was amplified using a 45bp homology with the ORF to be disrupted so as to increase the specificity for recombination. This product was transformed into diploid yeast strains and selected for G418 resistance. These transformants were sporulated and haploids of both mating types MAT*a* and MAT*a* were recovered from the tetrads. The deletions were further confirmed by PCR (Winzeler *et al.*, 1999). Each strain harbors replacement of one non-essential gene by KANMX cassette thereby conferring it G418 resistance. The KANMX cassette also carries molecular bar code on either side allowing differentiation of individual deletion strains in growth assays. Presence of two bar codes only adds to the specificity of the process. The tremendous strength of this collection is clearly evidenced by the increasing number of studies (including this one) that has lead to identification of previously unimagined connections between different processes (Winzeler *et al.*, 1999).

Our goal was to employ the entire deletion collection to uncover genes involved in targeting proteins to the INM. We needed to overcome two obstacles to reach this goal. First, we had to identify an easily detectable reporter protein residing at the INM. Trm1-II appeared to be an ideal candidate. We employed a fusion protein that contains GFP fused to Trm1-II after the last amino acid (aa) codon. Trm1-II-GFP locates to the nuclear rim exactly as endogenous Trm1-II (Fig.8; Rose *et al.*, 1992; Rose *et al.*, 1995) and its location is easily assessed in living cells when expressed from a low copy plasmid. The second obstacle was a means to introduce the plasmid into ~4850 members of the deletion collection.

Plasmiduction and 96 well Transformation

We initially attempted to introduce the plasmid by two different means: transformation in a 96 well format and plasmiduction in a 96 well format (Duchner, 1981). Plasmiduction approach involves mating a kar1-1 strain carrying a plasmid of interest with a KAR1 strain. kar1-1 causes a nuclear fusion defect, significantly decreasing the efficiency by which the two nuclei of a zygote fuse to form a diploid cell (Rose and Fink, 1987). These cells are capable of budding but they give rise to haploid progeny carrying the genetic material of one of the mating strains rather than a diploid offspring. At a very low rate (Dutcher, 1981) the plasmids harbored by one nucleus of the heterokaryon, in our case kar1-1 strain, are transferred into the other nucleus (plasmiduction) and inherited by the daughter cells during budding. These KAR1 cells harboring the plasmid can be specifically selected by using appropriate auxotrophic selection methods. The strategy of plasmiduction is depicted in Fig.6.

We tried to adopt this method to introduce the Trm1-II-GFP encoding plasmid (pAP1) into the strains of the deletion collection. We used $URA3^+$ kar1-1 strain carrying a *LEU2*+ plasmid expressing Trm1-II-GFP (kar1-1+pAP1). This strain was mated with *ura3*⁻ strains of the deletion collection. After mating the deletion strains with kar1-1 +pAP1, the deletions containing pAP1 plasmid were selected plating in media lacking leucine (selects for transformants containing the plasmid) and containing 5-Fluorootic Acid (5FoA) to select against kar1-1. This approach yielded plasmiductants (Fig.7). In

Figure 6: Plasmiduction Strategy, *kar1-1* harboring the plasmid pAP1 was mated with the deletion collection strains (96 well plate format). Plasmid is transferred from *kar1-1* to the other strain in a heterokaryon zygote. The two halves of the heterokaryon behave as haploid cells and give rise to daughter cells. The strains harboring deletions are selected by growing them on appropriate auxotrophic media.



Figure 7: Plasmiductants selected on auxotrophic Plasmiduction approach exhibited the transfer of plasmid from *kar1-1* to the strains of the deletion collection.



Figure 8: Establishment of a system to conduct a genome-wide screen for defects in targeting proteins to the INM. (A) Transformation in the 96-well plate format. To show transformation efficiency yeast cells in wells were pinned to solid media; left, mock transformation; right, transformation with pRS415-TRM1-II-GFP. (B) Trm1-II-GFP associates with the nuclear rim. BY4741, parent to the *MATa* deletion collection, was transformed with pRS415-TRM1-II-GFP and live cells were viewed by fluorescence microscopy. Scale bar = 5um.







our case, the plasmiductants are deletion strains containing the Trm1-II-GFP expressing plasmid. However, there were many disadvantages to using this approach. First, the efficiency of this approach is low (Dutcher., 1981) and secondly though at a low rate, chromosomes from the *kar1-1* strain can be transferred into the other partner and this would be undesirable for our studies. Therefore we decided to apply the process of transformation in a 96 well plate format for plasmid introduction.

As the plasmiduction approach does not provide an easy method for the introduction of our reporter into each member of the deletion collection, we adapted the one-step transformation protocol (Ito et al., 1983) to the 96-well format (Materials and Methods). Employing this procedure ~90% of the wells contain transformed cells (Fig. 8, right) with no background growth of cells from mock transformations (Fig. 8, left). Strains not transformed in the 1st round were re-transformed in a 2nd round; nearly all the 4850 strains were transformed. The location of Trm1-II-GFP in each strain was visually monitored by fluorescence microscopy

Predicted Mutant Phenotypes

Based on our knowledge at the time the screen was initiated we predicted to identify at least 5 different mutant phenotypes caused due to deletion in genes involved in targeting proteins to the INM. These are as described below.

1) Trm1-GFP present in the nucleoplasm

Figure 9: Mutant phenotypes anticipated in the screen A) Accumulation of Trm1-II-GFP in the nucleoplasm, B) Irregular distribution of Trm1-II-GFP around the NM leading to protein visualization in the shape of half moons or sickles, C) Overflow of the fluorescent protein into the ER, D) Protein mislocalized uniformly throughout the entire cell encompassing both nucleus and cytoplasm, E) Retention of nucleus within the cytoplasm and exclusion from nucleus probably due to inability to be transported across the membrane, F) Mis-targeting and accumulation of Trm1-II-GFP within cytoplasmic vesicles.



The wild type protein is tethered to the INM. Disruption of this interaction by deletion of the gene encoding the tethering partner would cause Trm1-GFP to accumulate in the nucleoplasm (Fig.9A).

2) Irregular distribution on the INM

Trm1-GFP is seen to be uniformly distributed throughout the INM to form a smooth ring /doughnut shape. However, mutations in genes that are involved in maintenance of the nuclear architecture or nuclear membrane organization maydisrupt the general structure of the membrane. This could lead to clustering of Trm1-GFP to form sickles that involve distribution of the protein only half way along the membrane or Trm1-GFP may be sequestered at one single spot to form speckles (Fig.9B)

3) Distribution in the ER

It has previously been shown (Stauffer *et al.*, in prep) in the lab that overexpression of Trm1 can cause the protein to be translocated into the ER owning to the continuity between the ER and the NM (Holmer and Worman, 2001). Defects in the INM targeting process, tethering of Trm1 to the INM or defect in the structure of the INM may cause the protein to be mislocalized into the ER. This phenotype may also be caused by saturation of the nuclear tether protein by overexpression Trm1 (Fig.9C).

4) Trm1-GFP in the cytoplasm or uniformly distributed within the cell

Many mutations may cause this defect. For example, mutations in pathways responsible for import of Trm1-GFP into the INM. Defects in gene encoding the

karyopherins, adaptors, NPC component may prevent the movement of Trm1-II-GFP across the membrane. This phenotype may also be the result of a defect in regulation of sorting and/or transport of proteins (Fig.9D and Fig.9E).

5) **Punctate distribution in the cytoplasm**

This could happen if the protein targeting machinery of the cell is defective and Trm1-II-GFP instead of being targeted to the INM mislocalizes within cytoplasmic vesicles (Fig.9F).

RESULTS

The NatC N-terminal acetyltransferase complex is essential for targeting Trm1-II-GFP to the INM:

Defects in gene products involved in nuclear import, nuclear structure, and targeting/tethering to the INM could lead to Trm1-II-GFP mislocation. We predicted that defects in the latter would make Trm1-II-GFP nucleoplasmic. Deletion of *MAK31* (*mak31* Δ) was the first mutation we found that caused Trm1-II-GFP to become nucleoplasmic (Fig.10A). The compound 4', 6'- diamidino-2-phenylindole dihydrochloride (DAPI) forms fluorescent complexes with dsDNA and is often used to demarcate the nucleus of a cell. The DAPI stain overlaps completely with Trm1-II-GFP in *mak31* Δ strains, further verifying the nucleoplasmic localization of the protein (Fig.10B).

A large number of strains in the deletion collection possess unintended genetic alterations (Hughes *et al* 2000) such as aneuploidy. It was therefore necessary to verify that the mutant phenotype we observe was due to deletion of *MAK31* gene and not any

Figure 10: Localization of Trm1-II-GFP mutants encoding components of NatC. *mak3* Δ , *mak3* Δ and *mak10* Δ (A) Top row: Trm1-II-GFP localizes to the nuclear rim in wild type and mislocalizes in the nucleoplasm in *mak31* Δ , *mak3* Δ , *mak10* Δ , Middle row: complementation of *mak31* Δ , *mak3* Δ and *mak10* Δ with plasmids encoding GST-tagged versions restores Trm1-II-GFP rim localization (B) Nuclear Stain Cells containing pRS415-TRM1-II-GFP were stained with DAPI (1 µg/ml) to show co-localization of Trm1-II-GFP with nuclear DNA. Top, GFP; bottom, DAPI. Scale bars = 5um.





other secondary effects. In order to confirm this, we complemented the *mak31* Δ cells expressing Trm1-II-GFP with another plasmid expressing GST-Mak31. This plasmid was isolated from a collection yeast strains, each containing a plasmid with a known yeast gene tagged with GST (Martzen *et al.*, 1999). As a control *mak31* Δ cells were also transformed with the vector alone. The nuclear rim localization was restored in cells expressing GST-Mak31 (Fig.10A). However, the vector alone control, showed no difference in nucleoplasmic localization of Trm1-II-GFP. This proves unequivocally that deletion of the gene *MAK31* results in mislocalization of the protein Trm1-II-GFP.

Mak31 is a subunit of the heterotrimeric NatC complex. NatC is an N-terminal acetyltransferase (NAT) and catalyzes the addition of an acetyl group to the N-terminus of a subset of proteins. In addition to Mak31, Mak3 - the catalytic subunit, and Mak10 make up the complex and are required for activity (Review: Polevoda and Sherman 2003). This information led us to "jump ahead" in our systematic screen of the deletion collection to study the consequences of $mak3\Delta$ and $mak10\Delta$ upon the subnuclear distribution of Trm1-II-GFP. We found that deletion of MAK3 or MAK10 also mislocates Trm1-II-GFP to the nucleoplasm (Fig.10A). We verified that the nucleoplasmic accumulation of Trm1-II-GFP is caused by deletion of MAK3 or MAK10 by complementation with plasmids encoding the corresponding genes using the yeast genomic GST-tagged collection (Fig.10A). The data show that all three of the NatC subunits are necessary to target Trm1-II to the INM.

Trm1-II-GFP was also nucleoplasmic in YPR050C deletion strain (data not shown). The putative YPR050C overlaps with *MAK3* and causes a phenotype similar to

Figure 11: NatA and NatB do not affect the localization of Trm1-II-GFP. Trm1-II-GFP localizes to the INM in *ard1* Δ and *nat1* Δ , deletion mutants of genes encoding the catalytic components of NatA and NatB respectively


that caused by NatC depletion. We surmise that inappropriate localization of Trm1-II-GFP in $YPR050C\Delta$ is actually due to MAK3 deletion.

Trm1 INM localization depends specifically on NatC

The yeast genome encodes for at least four NAT complexes, each with different substrate specificity. These are NatA, NatB, NatC and Nat4. Nat2, an essential gene was previously considered to be an N-terminal acetyltransferase. However, recently it has been revealed not to possess this activity (F.Sherman personal communication). NatA is comprised of two proteins Ard1 and Nat3 of which Ard1 is the catalytic subunit. NatB is comprised of two subunits Nat1, the catalytic subunit, and its partner Mdm20.

It is possible that Trm1-II-GFP INM mis-localization in $mak31\Delta$ cells is a secondary effect seen due to a general defect in the N-terminal acetylation of proteins within the cell. In this case, deletion of the catalytic subunits of both NatA and NatB complexes should have an effect on Trm1-II-GFP localization. However, deletion of neither *ARD1* nor *NAT1* had any effect on the INM localization of Trm1-II-GFP (Fig 11). The data indicate that Trm1-II-GFP mislocation in NatC mutants is not caused non-specifically by N-acetylation defects.

mak3A does not affect NM structure

Trm1-II-GFP could fail to associate with the INM in NatC-deficient cells either because NatC deficiency causes a general defect in nuclear membrane organization or **Figure 12:** *mak3* \varDelta does not affect Nsp1 localization. IF for Nsp1 shows punctate distribution surrounding DNA in wild-type and *mak3* \varDelta strains. Top: Nsp1 staining; bottom, merge of Nsp1 and DNA locations in the respective strains. Scale bar = 5um.



Figure 13: Ice2 is important for the INM location of Trm1-II-GFP but not for the nucleoporin, Nsp1. (A) Location of Trm1-II-GFP: Trm1-II-GFP is nucleoplasmic in two independent *YIL090W* Δ */ice2* Δ strains and *ICE2-GST* restores location of Trm1-II-GFP to the INM in the *MATa YIL090W* Δ */ice2* Δ strain. (B) IF for Nsp1: nuclear pores are not affected by *ice2* Δ . Top, IF for Nsp1 showing punctate distribution; bottom, merge of Nsp1 and DNA locations in the respective strains shows that Nsp1 surrounds the nucleoplasm. Scale bar = 5um.





because NatC activity is required for tethering a subset of proteins to the INM. To differentiate between these two possibilities, we investigated whether NatC is important for subnuclear distribution of a different class of nuclear rim proteins, nucleoporins. Nsp1 is an essential component of the NPC. Employing an Nsp1-specific antibody (Tolerico *et al.*, 1999) we determined the location of this symmetrically distributed nucleoporin in wild-type and NatC-deficient cells. Although the NatC complex causes redistribution of the majority of the Trm1-II-GFP pool to the nucleoplasm, it has no apparent effect upon Nsp1 location (Fig.12). Thus, there is no gross defect in nuclear membrane structure in NatC mutants.

YIL090W/Ice2 is required for INM targeting:

Screening of the deletion collection led to the discovery of another gene that possibly is involved in targeting Trm1-II-GFP to the INM. When we identified this gene to be important for Trm1-II-GFP subnuclear targeting, it was as yet uncharacterized and was assigned an ORF name YIL090W. However, de Martin *et al* (2005) recently reported that the protein encoded by YIL090W is involved in inheritance of cortical ER, thereby giving it the name *ICE2* (Inheritance of Cortical ER). Deletion of *YIL090W/ICE2* resulted in a Trm1-II-GFP nucleoplasmic pool, although for this mutant there is residual Trm1-II-GFP at the nuclear rim (Fig.12A, top row). To verify that *ice2* Δ is the cause of Trm1-II-GFP becoming nucleoplasmic, we transformed the Trm1-II-GFP encoding plasmid into a strain with an independently generated deletion (*MAT* α deletion collection) and found increased nucleoplasmic Trm1-II-GFP in the *MAT* α *YIL090W* Δ *ice2* Δ strain (Fig.13A). We also generated a new C-terminal GST-tagged Ice2 and expressed this tagged protein in ice2 Δ strains expressing Trm1-II-GFP. INM location is restored by *ICE2*-GST (Fig.13A). The data confirms that Ice2 is important for appropriate distribution of Trm1-II-GFP to the INM.

Trm1-II-GFP could poorly associate with the INM in *ice2* Δ cells because of a general defect in the nuclear membrane organization or because of a defect in tethering a subset of proteins to the INM. To distinguish between these possibilities, we assessed whether *ice2* Δ affects the subnuclear distribution of Nsp1. No effect was detected (Fig 13B). The data support a role for Ice2 in targeting/tethering a subset of proteins to the INM.

Ice2 localization

Analysis of Ice2 sequence predicts the presence of transmembrane domains. Different web based programs such as TMred, *Saccharomyces* genome database (SGD), differ in the prediction of the number of transmembrane domains. A closer look at the predictions by these algorithms indicated that some transmembrane domains are separated by a stretch of amino acid too short to form a loop connecting two transmembrane domains. We eliminated such short stretches of non-transmembrane regions and this lead to the prediction that Ice2 has at least 7 transmembrane domains which is in concurrence to the prediction of de Martin *et al* (2005). It is a Type II integral membrane protein; i.e. the amino terminal of the protein is predicted to face the ER lumen while the carboxyl terminal is towards the cytoplasmic side. C-terminal tags can interfere with nucleoplasmic anchoring allowing INM proteins to move to the contiguous

ER (Holmer and Worman 2001), however, since YIL090W/Ice2 with a C-terminal GST tag complements $YIL090W\Delta\Lambda ce2\Delta$ (Fig.13A), we effectively eliminated the possibility that the ER location of the tagged versions is incorrect.

Huh *et al* (2003) created an arrayed genomic library in which >90% of the yeast ORFs are tagged chromosomally with GFP. This enables localization of the proteins encoded by each ORF. The protein encoded by the ORF YIL090W localizes to the ER. How does a protein that localizes to the ER actually affects the localization of a protein targeted to the INM is a subject for further study.

DISCUSSION

The yeast deletion collections provide powerful tools to screen systematically for roles of all unessential proteins affecting any process of interest. Here we employed the collections in the first cell biology study to understand mechanisms by which nuclear proteins achieve their appropriate subnuclear distribution, focusing on the INM. This easy screen could be applied to any cellular location for which there exists a known resident protein. A priori one might have expected that proteins involved in organization of important structures, such as the INM, would be essential, prohibiting gene discovery using strains missing only unessential genes. However, we succeeded in identifying ORFs representing two different processes using the unessential yeast collections. A collection of the temperature sensitive mutants in essential genes is under construction (Boone, personal communication). When this collection is completed the role of every veast gene in targeting to any subcellular location can be learned using a similar strategy. We show that Trm1-II-GFP is not associated with the INM in strains lacking NatC activity. Our data provides the first evidence that N-terminal acetylation may be involved in targeting of proteins or tethering of a peripheral protein to the INM. We also show that $mak3\Delta$ does not affect the structure of the NM. Therefore, the mislocalization of Trm1-II observed in $mak3\Delta$ is not the result of an abnormal NM. These findings led us to propose alternative hypothesis to test the role of NatC in targeting of Trm1-II to INM. First, Trm1-II may be acetylated at the N-terminus and this modification is essential for its tethering to the INM, Second, the nuclear tether of Trm1-II which may be an integral INM protein is N-acetylated, Third, NatC plays an indirect role in targeting of Trm1-II to the INM. Chapter 4 discusses the various studies we carried out to test the above mentioned hypotheses and to better understand the part NatC plays in targeting Trm1-II to its physiological location.

We also unearthed, YIL090W, recently uncovered as Ice2 important for the structure of the cortical ER (de Martin *et al.*, 2005). YIL090W/Ice2 is an integral membrane protein with multiple transmembrane domains and GFP-tagged versions reside in the ER, rather than the INM (de Martin *et al.*, 2005, Huh *et al.*, 2003). Although C-terminal tags can interfere with nucleoplasmic anchoring allowing INM proteins to move to the contiguous ER (Holmer and Worman 2001), we show here that a YIL090W/Ice2 with a C-terminal GST tag complements the YIL090W Δ /*Ice2* Δ , effectively eliminating the possibility that the ER location of the tagged versions is incorrect. We also eliminated the possibility that YIL090W Δ /*ice2* Δ disrupts nuclear membrane structure because nuclear pores appear to be unaffected by deletion of this gene (Fig. 13B). We

are considering two alternative explanations for why Trm1-II-GFP is partially released from the INM in *ice2* Δ cells. First, it is possible that Ice2 resides in both the ER and INM and thereby serves as a tether for ER and INM proteins in each location. More likely, Ice2 is an ER component that indirectly affects Trm1-II subnuclear distribution perhaps by directing authentic tether(s) to the INM. It will be interesting to learn whether Ice2 plays an important role in targeting/tethering other proteins to the INM. The role of Ice2 in INM targeting is further addressed in Chapter 5.

CHAPTER 4

ABSTRACT

In the preceding chapter, we reported conducting a genomic screen to identify genes involved in targeting proteins to the INM. This effort led to the discovery of four genes unknown to be involved in the process of sorting proteins. Three of the genes MAK3, MAK31 and MAK10 encode products that form a complex with N-terminal acetyltransferase activity while the fourth gene ICE2 is involved in maintenance and inheritance of cortical ER. Mass spectroscopic analysis indicates that Trm1-II-GFP is Nacetylated. N-terminal mutations of Trm1-II-GFP predicted to ablate N-acetylation cause nucleoplasmic location, whereas a variant with an N-terminal alteration predicted not to allow N-acetylation by NatC is located at the INM, providing genetic support that Trm1-II N-acetylation is necessary for its subnuclear INM location. However, because Nacetylation appears not to be sufficient for INM targeting, it may provide a necessary role for INM targeting by affecting Trm1-II-GFP structure and exposure of cis-acting INM targeting motifs. Crosslinking of tagged Trm1-II to its neighbors on the INM revealed its association with a ~35KD protein. The protein does not cross-link with Trm1-II-GFP in the mak3 Δ mutants that display a defect in Trm1-II INM tethering. In this chapter, we describe the discovery of a novel role for a well known modification. We also report the identification of a possible nuclear tether of Trm1-II and maybe other INM proteins.

INTRODUCTION

Compartmentalization of different cellular processes to different organelles requires that there exist a mechanism for sorting of proteins. All proteins are synthesized in the cytoplasm and are then translocated to different regions of the cell. Signals present within the protein such as the MTS, NLS, PTS, etc are responsible for targeting proteins to mitochondria, the nucleus or peroxisomes, respectively. In addition to encoded cisacting peptides or structures post-translational modifications can play roles in directing proteins to the correct subcellular compartments. For example, both glycosylation and mono ubiquitination target proteins to membranes (Clark *et al.*, 2005). Other modifications like phosphorylation, acetylation, methylation have been implicated in different functions involving activation of proteins, silencing of chromatin, enabling protein-protein interaction and so on. Here, we provide evidence that N-terminal acetylation plays an as yet unexplored function in targeting proteins to the INM.

N-terminal acetyltransferases (NAT)

N-terminal acetylation is the modification of the alpha amino acid at the Nterminus of a protein using acetyl-coA as the acetyl donor. Internal acetylation of lysine especially in case of histones is a well studied subject. However, the functions of Nterminal acetylation are not well known. This modification is carried out by enzymes referred to as N-terminal acetyltransferases or NATs and is the most common modification in eukaryotes occurring in nearly 80-90% of the proteins in mammalian cells and nearly 50 % in yeast (Polevoda and Sherman., 2003), but rarely in prokaryotes or archaeal proteins.

The yeast genome encodes for at least 3 NATs, NatA, NatB and NatC. NatA is a heterodimeric complex of Ard1 and Nat3; NatB is a complex of Nat1 and Mdm20 while NatC is a heterotrimeric complex of Mak3, Mak10 and Mak31. The proteins Ard1, Nat1 and Mak3 are the catalytic subunits of NatA, NatB and NatC respectively (Polevoda et al., 1999). These catalytic subunits are part of the GNAT (GCN5 related Nacetyltransferase) family of N-acetyltransferase which includes more than 50 members. This family is characterized by the presence of 4 conserved motifs A-D (Vetting et al., 2005). These motifs are probably involved in binding to acetyl CoA and transferring of acetyl group to the N-terminus of the protein, as this is the only common property among the proteins in this group. This motif is also conserved in histone N-acetyltransferase (Hat1) and is characterized by the presence of the sequence Q/RxxGxG/A. Point mutations in any of the conserved motifs reduces the activity of Mak3 (Tercero and Wickner, 1992). Mak3, Nat3 and Ard1 form a sub-family within the GNAT family and thereby exhibit more similarity among themselves than with the other members. The noncatalytic partners of the NAT complexes, Nat1, Mdm20, Mak10 and Mak31 do not share similarity to the GNAT members.

The non-catalytic members of the NatC family, Mak10 and Mak31 reside in the cytoplasm whereas the catalytic subunit Mak3 has been localized to both the cytoplasm and nucleus (Huh *et al.*, 2005). N-terminal acetylation of proteins is believed to be co-

translational and should therefore occur in the cytoplasm. However, this has not been proved unequivocally.

Physiological Role of the NATs

The physiological roles of N-acetylation on native proteins are poorly understood despite considerable research efforts. None of the N-acetyltransferases are essential for cell viability. Since double deletions have not been carried out, it is not known if they are all dispensable together or share essential protein modification functions. Components of the NATs were discovered in various different genetic screens. The phenotypes of *ard1* Δ , *mak3* Δ and *nat3* Δ have long been scrutinized for clues as to the roles of N-acetylation. Each of these deletions has characteristic phenotypes that are exhibited by mutations in genes encoding other components of the complex. However, one common phenotype across all the NAT mutations is their inability to grow on non-fermentable carbon sources such as glycerol (Polevoda and Sherman, 2000).

NatA is a major NAT in yeast and acetylates a variety of proteins (Polevoda and Sherman, 2000). An elegant study (Gautschi *et al.*, 2003) revealed that interaction of Nat1 with nascent polypeptide chains occurs as the protein is being synthesized on the ribosome. These peptide chains are around 20-50 amino acids and as they emerge from the ribosomes, they are presented to the catalytic subunit Ard1 for co-translational N^{α} acetylation of the proteins. NatA is known to N-acetylate many ribosomal proteins, proteins involved in chromosomal silencing as well as unknown proteins likely to be involved in general growth of the yeast cell. Not surprising, *nat1* and *ard1* mutants exhibit the widest range of defects. These mutants demonstrate temperature sensitivity, derepression of $HML\alpha$ transcription, failure to enter G0, defects in sporulation, and salt sensitivity (Polevoda and Sherman, 2003).

Homologues of Nat1 and Ard1 exist in genomes of all model organisms, and they have been implicated in many vital processes including tissue development, cell proliferation etc (Choi *et al.*, 2001, <u>Sugiura</u> *et al.*, 2003). For example, in *Xenopus laevis*, the Nat1 orthologue, Xat-1, has been shown to be important in early embryonic development (Choi *et al.*, 2001); similarly the mice orthologue, *mNAT1*, is essential for development of the brain and neuronal proliferation and migration (<u>Sugiura</u> *et al.*, 2003). High expression of the human homolog, *NAT1*, has been associated in various cancers and the encoded protein has been suggested to play a role cell cycle control. Studies in higher organism have revealed occurrence of various defects due to mutations in the homologues of NatA subunits; however, neither the identity of the culprit substrates of N-acetyltransferase nor the mechanisms by which mutations in Nat1 confers the variety of defects observed, are known.

The yeast NatB acetyltransferase contains the catalytic subunit Nat3 and Mdm30. Mutations in either of the subunits results in slow growth, osmotic sensitivity, reduced mating efficiency, inability to form functional actin cables, defects in inheritance of mitochondria, polarity of buds and sensitivity to DNA damaging agents (Polevoda and Sherman, 2003). Some of these phenotypes may be explained by the identity of NatB substrates, which include proteosomal subunits Pre1, Rpt3, and Rpn11, ribonucleotide reductase Rnr4 and ribosomal proteins S21 and S28. NatB also N-acetylates tropomyosin; this modification is essential for its interaction with actin (Singer and Shaw,

2003), which, in turn, causes defects in movement of organelles such as mitochondria from the mother to the emerging daughter bud. Both Nat3 and Mdm20 are conserved proteins and homologues have been identified in *Arabidopsis thaliana*, *C.elegans*, *D. melanogaster*, *X. laevis* and *Homo sapiens* (Polevoda *et al.*, 2003).

NatC components were first identified as being important for the maintenance of the L-A double stranded (ds) RNA killer virus in yeast and were named for their activity as maintenance of killer (MAK) (Tercero et al., 1992, Polevoda and Sherman, 2001). NatC N-acetylates the viral coat protein, gag, and this modification is vital for assembly of the virus particles. The mak3, mak10 and mak31 mutants display growth defects on non-fermentable carbon sources. Many proteins targeted to the mitochondria, like α ketoglutarate dehydrogenase (Kdg1), fumarate dehydratase (Fum1) and a mitochondrial ribosomal protein (Mrp1) possess N-terminal sequences that potentially can be recognized and acetylated by NatC (Polevoda and Sherman, 2001). They may be mislocalized or lack respective activities in the absence of this modification, which could explain the inability of the mutants to grow on non-fermentable carbon sources. However, no biochemical study has shown that these proteins are NatC substrates. In fact known substrates of NatC are relatively rare. Among the proteins identified to be NatC substrates are the proteosomal subunits Pup2 and Pre5 (Lee et al., 1989), a small GTPase targeted to the golgi Arl3 (Behnia et al., 2004, Setty et al., 2004) and as presented in this chapter, the tRNA methyltransferase, Trm1.

Specificities of NATs

N-terminal acetylation may occur either co-translationally or post-translationally. Acetylation carried out by the NATs is believed to be a co-translational process in eukaryotic cells but occurs post-translationally in prokaryotes. In vitro studies indicate that N-acetylation of proteins occurs when there are between 25-50 residues extruding from the ribosome (Driessen et al., 1985). This has been shown to be true for the NatA enzymes (Gautschi et al., 2003); however, similar studies have not been conducted for the other Nats. Each of the Nats recognizes a subset of proteins with specific N-terminal sequences. NatA recognizes the more abundant proteins with N-termini, -ser, -ala, -gly or -thr. The initiator methionine is cleaved off in a co-translational manner from a protein when penultimate residues have radii of gyration 1.29A or less. These include the amino acids glycine, serine, proline, threonine, alanine, cysteine and valine. This hypothesis has been confirmed by Polevoda and Sherman using altered yeast iso-1-cytochrome C with all possible penultimate amino acid combinations (Polevoda and Sherman, 2003). The same study also confirmed the sequence requirements of NatB and NatC. The N-termini commonly recognized by the NatB complex includes Met-Glu, Met-Asp and Met-Asn. In contrast, NatC acetylates a subset of proteins with the termini, Met-Leu, Met-Ile, Met-Phe or Met-Trp. None of these are steadfast rules and the process of N-acetylation seems to require other as yet unknown sequences or structures as not all proteins that possess the defined N-terminal sequences are actually acetylated (Polevoda and Sherman, 2003).

Motifs sufficient for INM targeting

A parallel study on Trm1 carried out by Karen Stauffer led to the identification of a region within the Trm1-II protein sequence that is sufficient for targeting reporter proteins to the INM. This region encompassing the motif referred to as ADEPT2 (Stanford *et al.*, 2000) targets an ectopic protein β -galactosidase to the INM (Stauffer *et al*; unpublished results). When ADEPT2 is expressed as a fusion protein with a different reporter, GFP (Adt2-GFP), the fusion protein is targeted to a single spot on the NM similar that observed in case of SPB components. SPB components have been reported to interact with peripheral INM proteins such as Mlp2 (Niepel *et al.*, 2006). We therefore suspected an interaction between Adt2-GFP and SPB component. We show here colocalization studies indicating that Adt2-GFP localizes to a spot on the INM distinct from the SPB. Finally, we propose working models of the mechanism by which Trm1-II is targeted and tethered to the INM.

RESULTS

A requirement for NatC in targeting Trm1-II-GFP to the INM could be explained by at least three mechanisms. First, Trm1-II could be a NatC substrate and its Nacetylation could function in INM targeting. Second, the Trm1 INM tether could be Nacetylated, enabling interaction with Trm1. Finally, N-acetylation could act indirectly. Since the first of these models requires that Trm1 be a NatC substrate and we conducted genetic and biochemical studies to learn whether this is the case.

N-terminal acetylation of Trm1

Inspection of the Trm1-II N-terminal sequence [all nomenclature here refers to aa starting at the 2nd AUG, i.e., ORF codon 17 is referred to as aa1] revealed the sequence Met-Leu-Lys-Ala which is a signature sequence of NatC substrates (Polevoda and Sherman 2003). We employed both genetics and biochemical methods to analyze whether Trm1 is acetylated and if this modification plays a role in its subnuclear localization.

Our genetic studies indicated that N-acetylation of Trm1-II may be important for its INM localization. So we reasoned that mutational changes on Trm1-II that prevent Nterminal acetylation would have the same effect as $mak3\Delta$. Therefore, we first attempted to carry out point mutations at the N-terminus of Trm1-II so as to prevent acetylation of the protein or to enable its recognition by other N-terminal acetyltransferases. Specificity of a protein to be recognized for N-acetylation seems to lie in the first 2-4 amino acids. The N-terminal sequences Met-Leu, Met-Trp, Met-Phe or Met-Ile are the amino terminal sequences recognized by the NatC complex (Polevoda and Sherman, 2001). Thus NatC recognizes a hydrophobic amino acid next to the initiator methionine. A well characterized physiological substrate of NatC in yeast is the gag protein encoded by the killer virus LA (Tercero et al., 1993) with the N-terminal sequence Met-Leu-Arg-Ala. Studies conducted using iso-cytochrome C synthetic substrates indicate that the presence of positive amino acid at position 3 increases the efficiency of N-acetylation by the NatC complex (Polevoda and Sherman, 2003). These studies indicate that the N-terminal sequence of Trm1, Met-Leu-Lys-Ala, to be an ideal candidate for N-acetylation.

Figure 14: Trm1-II-GFP may N-acetylated by Nat C. Wild type cells expressing the mutated protein Trm1-II-GFP ΔL_2 (left) exhibit nucleoplasmic localization of the protein. Cells expressing Trm1-II-GFP K₃ \rightarrow E₃ also cause Trm1-II-GFP to mislocalize. Thus, N-terminal mutations that inhibit N-acetylation prevent nuclear rim localization of Trm1.



Therefore, our initial attempt was to prevent N-acetylation of Trm1-II. Previous studies have shown that presence of a basic amino acid such as lysine prevents its recognition by any of the known Nats in yeast. This guided our decision to delete the three bases that code for Leu at the N-terminus of Trm1 thereby generating the N-terminal sequence of the protein Met-Lys-Ala. This protein should not be recognized and N-acetylated by any of the Nats. This mutant protein, Trm1-II-GFP ΔL_2 , when expressed in wild type cells localized to the nucleoplasm, in a manner similar to that observed in mak3d cells (Fig.14 left), consistent with the model that N-terminal modification by NatC is necessary for targeting/tethering to the INM. However, deletion of one amino acid, though unlikely, could cause a conformational change preventing binding of the protein to the INM. In this case lack of INM association would be due to the modified protein sequence rather than lack of N-acetylation. To address this caveat, we sought to prohibit N-acetylation without deleting any of the N-terminal amino acids. In case of gag protein of L-A virus, change of a positive aa arginine (R) at position 3 to glutamic acid (E) prohibits its Nacetylation (Tercero et al., 1992). We changed Trm1-II-GFP K₃ to E₃.

When Trm1-II-GFP $K_3 \rightarrow E_3$ was expressed in wild-type cells, it was located in the nucleoplasm (Fig.14 right). Thus, both mutations predicted to change Trm1-II-GFP from an ideal substrate to an unlikely NatC substrate caused its mislocation to the nucleoplasm. This provides genetic support for the model that Trm1-II-GFP is N-terminally acetylated. Our subsequent experiments were directed at biochemically detecting the presence of the acetyl group at the N-terminus of Trm1-II-GFP

Figure 15: Trm1-II is N-acetylated. (A) IF for wild-type cells expressing Trm1-II-GST. (B) SDS-PAGE of purified Trm1-II-GST, (lanes 1 and 2); marker proteins, (lane 3). (C) N-terminal peptides identified by MS; arrows indicate identified Glu-C cut sites. Scale bar = 5um.



Figure 16: Strains transformed with pGP-Trm1-II-GST express protein of correct molecular wt. Glutathione column, purified protein from wild type and $mak3\Delta$ strains were transferred on to polyvinylchloride membrane. The protein was detected by use of primary mouse anti-GST and secondary horse radish peroxidase (HRP) tagged secondary antibody.



- 1. Wild type cell lysate before purification
- 2. Wash buffer from the column
- 3. Purified protein eluate
- 4. Visual marker (not visible in western)
- 5. mak3∆ cell lysate
- 6. Wash buffer from the column
- 7. Purified protein eluate
- 8. Western Marker
- 9. Vector alone control cell lysate
- 10. Wash buffer
- 11. Purified protein eluate
 - Trm1-II-G ST (89KD)
 - Cleaved Trm-II-GSt
 - GST alone (25KD)

Purification of Trm1

The mutational studies described above support the model that Trm1-II is Nacetylated by NatC. Our next step was to biochemically prove the presence of an acetyl group at the N-terminus of Trm1-II. This could be established by the use of mass spectrometry. In order to carry out mass spectrometric analysis, we had to purify the Trm1-II protein in sufficient quantity so as to allow analysis of the N-terminus. All the commercially available constructs of the protein were constructed with Trm1-I, the long form of the protein located primarily in the mitochondria or were fused with the tag at the N-terminus (Martzen *et al.*, 1999) and they were not usable for our purposes. Therefore, we constructed a plasmid encoding a C-terminal GST tagged Trm1-II under control of a galactose promoter. This allowed us to over express Trm-II-GST facilitating purification of sufficient quantities of the protein for chemical analysis. Employing anti-GST and IF we determined that Trm1-II-GST is located predominately at the INM in wild-type cells (Fig.15A), but is nucleoplasmic in *mak3* cells (data not shown), as is Trm1-II-GFP.

Each preparation of purified Trm1-II-GST contained primarily a single protein that migrated on SDS gels as predicted for the full-length fusion protein (Fig.15B) and confirmed to cross-react with anti-GST by Western analysis (Fig.16). Previous studies showed that Trm1 has the same subnuclear location when over-produced (Rose *et al.*, 1992; Rose *et al.*, 1995), indicating that the INM tether for Trm1 is not limiting. Failure to co-purify other proteins could mean that Trm1 is tethered to an integral INM protein that is not soluble under our purification conditions, or to a peripherally associated protein that is lost during purification, or that the Trm1 tether is not a protein.

Mass spectrometry reveals that Trm1 may be N-terminally acetylated

Mass spectrometric analyses of Trm1-II presented many obstacles. The protease trypsin is the best characterized for use in in-gel digestion of the proteins. Trypsin recognizes and cleaves peptide bonds on the carboxyl side of a basic amino acid such as lysine or arginine and to a lesser extends cysteine. In Trm1-II lysine is present at position 3 of the peptide chain. Cleavage of this protein by trypsin gives rise to a peptide of molecular weight about 400 Da without N-acetyl modification and about 440 with the modification. This molecular weight is close to the lower detection limits of mass spectrometry. Our attempts to detect this peptide failed. However, we did succeed in detecting various Trm1-II peptides allowing unequivocal identification of the purified protein.

Another protease widely used in mass spectrometric analyses, is chymotrypsin. Chymotrypsin is a serine endopeptidase with specificity to hydrolyze at the C-termini of amino acids –Thr, -Phe, and –Trp. It also recognizes and cleaves peptide bonds at the C-termini of amino acids –Lys, -Met, -Ala, -Asp and –Glu at a lower rate. Hence, this peptidase would also generate to peptides close to the lower detection limit of the mass spectrometer, deterring our attempt to identify the N-terminal peptide. We therefore decided to employ an endopeptidase isolated from the bacterial *Staphylococcus aureus* (protease V8) Glu C. This enzyme cleaves peptide bonds C-terminal of glutamic acid (D) and with a 3000-fold lower rate at aspartic acid (E). We used buffer conditions that allow cleavage of both Glu and Asp. Examination of the sequence of Trm1 indicates that digestion by Glu C at C-terminus of glutamic acid would generate a 25 amino acid, 2730

Da unmodified peptide or 2777 da peptide when acetylated at the N-terminus (Fig.15C). We detected the 2777 da peptide in protein sample prepared from the wild type cells. MS information for Trm1-II-GST from $mak3\Delta$ cells was uninformative because we did not detect the 25 aa peptide, with or without the N-acetyl moiety, even though the adjacent downstream peptide starting at F₂₆ was present. Instead, we detected peptides that could be accounted for by limited N-terminal proteolysis (Fig.15C), perhaps indicative of N-terminal protection by the N-acetyl moiety, either in vivo or in vitro during protein purification. The biochemical studies show that Trm1-II bears an N-terminal acetyl group and support the model that it is a NatC substrate.

N-terminal acetylation is unlikely to be sufficient for INM localization

The biochemical studies indicated that the N-terminus of Trm1-II-GFP is acetylated, and we have shown that N-acetylation is necessary for rim localization. We were now interested in determining if N-acetylation is the only requirement for INM association. We reasoned that if N-acetylation is sufficient to target Trm1-II to the nuclear rim, then, the addition of ectopic N-acetylation sequences to Trm1-II-GFP ΔL_2 would restore wild type localization of this mutant protein. We chose two different 4 aa peptide sequences that are identical to the N-termini of known substrates and added them to the Trm1-II-GFP ΔL_2 N-terminus (Fig.14 left). This 4 aa addition created M<u>FLTR</u>K, identical to the Pup1 N-terminus, a known NatC substrate, and M<u>SLPA</u>K, identical to the Rps0A/Yst1 N-terminus, a known NatA substrate (Polevoda and Sherman 2003). When expressed in wild type yeast cells neither Trm1-II-GFP variant located to the INM

Figure 17: N-terminal amino acids important for Trm1-II-GFP INM targeting. (A) Introduction of ectopic N-acetylation sequences at N-terminus does not restore INM location for Trm1-II-GFP Δ L₂. MSLPAK and MFLTRK, the NatA recognition sequence from Yst1 and the NatC sequence from Pup1, respectively, appended to the N-terminus of MKA. (B) Mutation predicted not to disrupt N-acetylation, MFKA, does not affect Trm1-II-GFP association with the INM in wild-type cells; however, this protein is nucleoplasmic in mak3 Δ cells. Scale bars = 5um.



A

B

(Fig.17A). This could mean that either the variant Trm1 proteins are not N-acetylated as predicted, or if they are, N-acetylation is not sufficient for INM targeting, even though it is necessary.

The N-terminal changes described above involve the addition of 4 amino acids and cause an extension of N-terminus. Such an extension may interfere with its interaction with the nuclear tether. Therefore, we chose to carry out conservative single amino acid changes. We changed Leu2 to Phe2, this protein Trm1-II-GFP $L_2 \rightarrow F_2$ localizes to the nuclear rim similar to the wild type. When expressed in *mak3A*, it is nucleoplasmic. Thus, Trm1-II-GFP $L_2 \rightarrow F_2$ behaves like the wild type protein (Fig.17B).

These mutational studies demonstrate that N-terminal acetylation by itself is necessary but may not be sufficient. Also, it seems that the N-terminal sequence of the protein and not just its modification is important. Trm1-II-GFP might be interacting with an INM protein via its N-terminus. Two proteins might interact in a manner similar to enzyme and substrate where the latter fits into a groove, socket or pocket of the enzyme. The interaction between the amino acid in the enzyme and substrate depends on the presence of particular amino acid side chains and the charge. This would explain why the substitution of a negatively charged hydrophilic amino acid (Glu) for a hydrophobic amino acid (Leu) mislocalized the protein in spite of preserving the substrate recognition sequence for N-acetylation. It also explains why a conservative substitution of hydrophobic Phe for Leu does not affect INM location of Trm1-II-GFP. **Figure 18**: Crosslinking strategy for isolation of membrane protein tethering Trm1-II to the INM



Figure 19: Crosslinking of Trm1-II-GST to nuclear membrane proteins to identify the tether of Trm1-II. The overexpression of Trm1-II-GST results in highly non-specific crosslinking of the protein disabling the identification of bands missing in $mak3\Delta$


- 1. Crosslinked protein purification from wild type cells
- 2. No crosslinker preparation from wild type cells
- 3. No crosslinker preparation from *mak3*∆ cells
- 4. Crosslinked protein preparation from mak3∆ cells
- 5. Marker

Crosslinking studies provide clues for nuclear tether for Trm1

Trm1-II is most likely tethered to the INM via protein-protein or protein-lipid interactions. Our purification of Trm1-II-GST did not co-purify any proteins associated with it, perhaps indicating that Trm1-II may interact with an integral membrane protein which is not isolated by our non-detergent based methods of protein purification. Previous methods to identify Trm1 interacting proteins by 2-hybrid have proved to be futile (Hopper, personal communication). However, the standard 2-hybrid method rarely uncovers integral membrane proteins. To learn whether Trm1-II is targeted to the INM via association with other peripheral or integral INM proteins we elected to employ *in vivo* crosslinking.

Chemical reagents with two reactive groups can be used to cross link proteins close to each other within a cell. The protein complex can be purified and the co-purified proteins separated to identify the interactors. We carried out *in vivo* crosslinking to bind a tagged Trm1 to its tether and purified the protein complex. We predicted that a biologically relevant protein would be present in the complex purified from the wild type strain but absent in $mak3\Delta$ (stratergy represented in Fig.18), in which Trm1 does not interact with its nuclear tether. Initially we used a galactose inducible Trm1-II-GST for this purpose. Galactose induction results in nearly 30-fold over expression of the protein. We thought this would facilitate purification of Trm1-II interactors at high concentration.

A disadvantage of overexpressing Trm1-II-GST is that crosslinking is highly non-specific and chemically binds neighboring proteins that may have no physical interaction in the cell. This combined with the fact that overexpression of Trm1-II-GST causes some of the protein pool to translocate into the ER (Stauffer *et al.*, in prep), resulted in a

Figure 20: Trm1-II-TAP crosslinking leads to recovery the membrane tether of Trm1-II. Trm1-II-TAP was crosslinked with DSP, and the protein purified from wild type and *mak3* Δ strains. Two different preparations yield identical results. The arrow indicates the presence of band observed in wild type and its absence in *mak3* Δ cells. Lanes 1 and 4: two separate preparations of crosslinked protein from wild type strain, Lanes 2 and 5: two separate preparations of crosslinked protein from *mak3* Δ strains

- Trm1-II-TAP crosslinking prep. From wild type cell
- mak3∆ cells
- Marker
- Wild type



- No crosslinker protein prepration from Wild type cells
- Crosslinked protein purification from wild type cells
- No crosslinker prepration from *mak3*∆ cells

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• Crosslinked protein preparation from mak31 cells



complex pattern likely including non-specific crosslinking of not only proteins present on the nuclear membrane but also the ER (Fig.19), thus, making it difficult to discern the differences between the wild type and the mutant.

A yeast-TAP-fusion library created by Dr. Weissman has each ORF tagged with the high affinity TAP epitope (Rigaut et al., 1999). The fusion protein is expressed from its native chromosomal location. This fusion library allows the purification and selection of the entire yeast proteome and associated components using simple affinity purifications. Studies carried out by this group determined that Trm1 is present in about 14000 copies within the cell. This relatively high abundance enabled purification of the protein without the need to over express it. The Trm1-TAP strain was purchased from Open Biosystems. The TAP-tag collection was generated in the strain background BY4741, the same strain used to generate the deletion collection. Trm1-TAP strain was mated with the mak3 Δ from the deletion collection (MAT α , BY4742) and the tagged TRM1 gene was segregated into the mak3 Δ by tetrad dissection. Trm1-TAP MAK3 and Trm1-TAP mak 3Δ strains were then employed in crosslinking experiments using DSP as the crosslinker. In contrast to our previous attempts for this procedure, we also used detergent and a high concentration of urea to dissolve the cell membranes and release integral membrane proteins. Two different experiments yielded a protein of around ~35KD PAGE present in the wild type protein preparation but absent in the mak3 Δ strain (Fig.20).

Trm1-TAP was purified from the wild type and mutant under two different conditions, (1) without the crosslinker and (2) after incubation with the crosslinker DSP.

Our rationale was that Trm1-II interactor would to be present in the wild type sample treated with DSP and not in the one processed without the crosslinker, while $mak3\Delta$ would lack this protein in both the preparations. Much to our surprise, we found the ~35KD band to be present in both wild type samples, however, it was in lower quantities in the sample not treated with DSP. The high concentration of detergents used to dissolve the membranes might be responsible for the release of the protein. It would explain why we could not detect this band in our previous purification attempts. The $mak3\Delta$ lacked this band in both of the differentially prepared samples.

The \sim 35KD band was isolated from the gel and mass spectrometric analysis to identify the protein is in progress.

Adept2 is sufficient for localization to a sub-region of the INM

A parallel study was conducted in our lab to deduce the cis-acting sequences within Trm1-II that are sufficient for tethering it to the INM. This study (Stauffer *et al.*, in prep), uncovered a domain, approximately 60 amino acids long, that is sufficient to target a reporter protein to the INM. This region, ADEPT 2, is one of the 4 ADEPTs identified within the Trm1 sequence (Stanford *et al.*, 2000). The identification of the role of ADEPT 2 in targeting of Trm1 not only validates the ADEPT hypothesis but also uncovers a novel sequence as important for determining sub-organellar locations of a protein. When fused with an ectopic protein β -galactosidase, ADEPT2 targets it to the INM (Stauffer *et al.*, in prep). However, when fused to another reporter, GFP (adt2-GFP), the fusion protein localizes as a single bright spot in the nucleus (Shaheen and Hopper

Figure 21: Adt2-GFP does not localize to the SPB. A strain expressing Spc42 as an RFP (left) fusion protein was transformed with a plasmid encoding Adt2-GFP under control of galactose promoter (Middle). Lack of colocalization of the RFP and GFP signals indicate that Adt2-GFP does not localize to the SPB (Right).



Wild Туре ВҮ4741

unpublished data, Fig.21). Overexpression of this fusion protein results in its accumulation throughout the nucleus providing support for our assumption that adt2-GFP localizes to the INM rather than ONM (Shaheen and Hopper, unpublished data).

Is ADT2-GFP at the SPB?

The fusion protein adt2-GFP is sequestered to one spot within the yeast nuclear membrane. This spot does not coincide with the nucleolus and overexpression of the protein causes it to escape into the nucleoplasm (Shaheen and Hopper, unpublished data) indicating its location at the INM. The components of the SPB stain as a bright spot similar to adt2-GFP. A recent study (Niepel et al., 2005) illustrates the importance of peripheral INM protein Mlp2 in NM insertion of SPB via interaction with SPB constituents such as Spc42, Spc110 and Spc29. Moreover, we have often observed the accumulation of Trm1-II-GFP, especially when overexpressed or expressed in endogenous quantities in a manner similar to SPB. This led us to propose that adt2-GFP might tether to some SPB components. To test this proposal, we employed a strain expressing a component of SPB, Spc42, as a red florescent protein (RFP) fusion (a kind gift from Erin O' Shea, UCSF). This strain was transformed with a plasmid encoding adt2-GFP. Adt2-GFP did not colocalize with Spc42-RFP. In majority of the cells, the adt2-GFP staining was distinct from that of Spc42-RFP (Fig.21). The data clearly indicates that adt2-GFP does not bind to any of the components of the SPB. Which component of the NM actually interacts with adt2 is yet to be determined.

DISCUSSION

Subcellular compartments contain specific proteins that establish their ability to carry out particular cellular functions. Often proteins may be found in more than a single organelle. Dual distribution may be achieved by alternative splicing of the same gene, gene duplication or two translation initiations on a single mRNA. In each case, the end result is production of different sets of proteins, each targeted to separate organelles. Proteins harbor targeting signals, such as MTS, PTS, NLS, ER signal retention peptides and so on. Once within the organelle, such as mitochondria, nucleus, ER, etc, the proteins may either integrate into the membrane or sequester to sub-organellar locations. Each step of the sorting process requires a mode of communication between the protein to be sorted and proteins involved in the process of sorting, as offered by the signal peptides or other signaling motifs.

Sorting of proteins to different organelles requires interaction with its surface receptors and translocation through the membrane (Munro, 2002, Karniely and Pines, 2005). Each organelle has an identity that is determined by the distinctiveness of the proteins on its surface (Munro, 2002). This uniqueness facilitates the interaction of these membrane proteins with newly synthesized proteins harboring the characteristics for entry that may be defined by sequence motifs. The targeted proteins may interact with the organelle surface directly or through adapters as often observed for transport to the nucleus. In either case, the specific or unique motifs within the protein to be sorted are required.

Sorting of proteins is often controlled by accessibility of the targeting sequence. This mode of control is common for a single protein harboring two different targeting signals or an ambiguous signal that may be recognized by more than one organelle trafficking machinery. Accessibility to targeting sequence is controlled by folding of the protein to conceal or reveal the signal, interaction with another protein, or conformational changes. Post translational modification may aid in conformational changes that expose the targeting sequence. For example, the protein CYP2B1 is distributed in the ER and mitochondria. Phosphorlation of an internal motif by protein kinase A (PKA) conceals the MTS allowing the protein to be sequestered to the ER (Anandatheerthavarada *et al.*, 1999).

Our attempt to understand the process by which Trm1-II is targeted to the INM led to identification of two previously inconceivable processes as being involved in this process, (1) N-terminal acetylation of proteins and (2) cortical ER formation. The genes involved in these processes are *MAK3*, *MAK10*, *MAK31* and *ICE2*, respectively. We have presented data in this chapter illustrating that N-terminal acetylation of Trm1-II is necessary for tethering it to the INM. Conservative changes of the N-terminus do not affect the localization of the protein. In contrast, predicted changes of substrate specificity of Trm1-II to NatA or NatB by altering its N-terminal sequence mislocalize the protein. Our data is consistent with Behnia *et al* (2004) who reported that the NatC-dependent N-acetylation is necessary for targeting Arl3 to the Golgi, but N-terminal alterations that allow N-acetylation of Arl3 by NatA or NatB motifs do not result in its Golgi distribution. Arl3 targeting is dependent upon its interaction with a golgi

membrane protein Sys1 (Setty *et al*; 2004 and Behnia *et al*; 2004). A change in the Nterminal sequence of Arl3 disrupts its interaction with Sys1. There are other reports of the necessary roles of N-acetylation in subcellular trafficking, For example, it has been reported that Sir3 termini that are modified by NatA function inappropriately (Wang *et al.*, 2004). Tropomyosin, a substrate of NatB, fails to bind efficiently with actin when unacetylated (Singer and Shaw; 2003). A study in *Arabidopsis*, demonstrated the necessity of N-acetylation of chloroplast precursor proteins for their efficient sorting to the organelle (Pesaresi *et al.*, 2003).

Ectopic NatC motifs do not restore the INM location of Trm1-II-GFPΔL₂. We propose three different explanations for apparently anomalous finding. The first is that the altered proteins are not acetylated even though they have ectopic Nat motifs. Second, appropriate subnuclear distribution could require both N-acetylation that is achieved with the ectopic sequence and a Trm1 specific N-terminal sequence or structure disrupted by the ectopic sequences. Third, the N-acetyl moiety might not interact directly with the INM, but rather it could regulate protein folding and exposure of other cis-located targeting motifs and the proteins with ectopic sequences may not achieve the appropriate structure. The latter possibility is consistent with the previous observation that a beta-galactosidase fusion containing the N-terminal one third of Trm1-II fails to associate with

Figure 22: Model to explain the role for N-terminal acetylation in targeting Trm1-II to the INM. N-acetylation confers a conformational change in Trm1, exposing the region that tethers Trm1 to the INM, ADEPT2. Trm1 interacts with its nuclear membrane tether via ADEPT2, this facilitates Trm1-Trm1 interaction through its N-terminal acetylated region leading to the formation of a chain of Trm1 molecules that spread around the periphery of the NM.



the INM (Li et al., 1989) and to the fact that the motif ADEPT2 is sufficient to target an ectopic protein to the INM. According to this model (Fig.22), N-terminal acetylation by NatC exposes the ADEPT2 region on Trm1-II, allowing it to interact with its membrane tether. In the absence of N-acetylation, the ADEPT2 region is not exposed and Trm1-II is unable to interact with the tether and accumulates in the nucleoplasm. Crosslinking of Trm1-II to its INM neighbors isolated a ~35 KD protein (NTT for putative nuclear tether of Trm) from the wild type samples but not from the $mak3\Delta$ cells. So Trm1-II interacts with NTT only when acetylated at the N-terminus. Once attached to NTT, the acetylated N-terminus of Trm1 interacts with other Trm1 molecules enabling it to spread around the INM. Such an interaction is supported by the identification of Trm1 as interaction with itself in genome wide 2 hybrid screens (Uetz et al., 2000). Ribas et al (1998) have reported such an interaction between viral protein Gag to form the capsid and this interaction is absent in *mak3* Δ mutants causing inability of the viruses to assemble. In a manner similar to the gag protein of killer virus, N-acetylated end of Trm1 could interact with internal motifs of other Trm1 facilitating the spread of Trm1 around the NM. This model would also explain the specificity of amino acids at the N-terminus as observed in ectopic changes.

We propose that when fused to an ectopic protein such as β -galactosidase or GFP, ADEPT2 is exposed for interaction with the tether and is therefore sufficient for targeting proteins to the INM. Adt2-GFP does not possess the ability to spread around the entirety of the NM. Our model (Fig.22) may explain the inability of Adt2-GFP to spread around the NM by attributing it to the lack of N-terminal regions of Trm1 required for

Trm1-Trm1 interaction and subsequent spreading. Adt2-GFP is observed as a bright spot on the INM similar to localization of SPB components. A recent study indicating the interaction between a peripheral INM protein Mlp2 and SPB components led us to propose the initial site of interaction between Trm1 and INM may at the SPB. Trm1 may interact with the SPB component via ADEPT2 and Trm1-Trm1 interaction could enable the spread of the protein around the boundary of the INM. This hypothesis would require that Adt2-GFP localize to the SPB. However, this spot does not co-localize with SPB as shown by the colocalization of Spc42-RFP with Adt2-GFP. Each of these fusion proteins localize to distinct locations on the INM.

Finally, at a time when the various functions of N-terminal acetylation are surfacing, such as targeting to golgi (Setty *et al*; 2003, Behnia *et al* 2003), chloroplast (Pesaresi *et al*; 2003), interaction with other proteins as in case of Sir3, we report the importance of this modification in targeting a protein (Trm1-II) to the INM (Murthi and Hopper., 2005). We also report the interaction of Trm1-II to a 35 KD NM protein in the presence of the N-terminal modification and its absence when Trm1-II lacks N-terminal acetylation

CHAPTER 5

ABSTRACT

My genomic screen for genes playing a role in targeting proteins to the INM or maintenance of the NM structure and biogenesis yielded a mutant carrying a deletion of the gene ICE2. ICE2 encodes an integral membrane protein present in both perinuclear and cortical ER and functions in the inheritance of cortical ER (de martin et al., 2005). The ER tubules are continuous with the ONM and these tend to elongate towards the cell periphery and push against the plasma membrane. The ER towards the NM is called the perinuclear ER while that near the plasma membrane is referred to as cortical ER. In this thesis, chapter I describes the investigation of the possible mechanisms by which Ice2 affects targeting Trm1-II-GFP to the INM. Ice2 seems to be uniquely encoded by the fungi family members as a homologous protein is thus far absent from higher eukaryotic genomes. Here we investigate the interaction of this ER protein with a microtubule depolymerizing protein Kar3. kar3 Δ has been reported to be synthetically lethal with *ice2* Δ (Tong *et al*; 2001). In addition to confirming the synthetic lethality, I show that $kar3\Delta$ and $ice2\Delta$ cells possess similar phenotypes such as elongated microtubules. The cytoskeletal component, microtubule has been previously shown to be involved in the targeting of organelles as well as proteins to various subcellular locations. So I investigated the role of cytoskeleton in targeting Trm1-II-GFP to the INM. We used both temperature sensitive mutants of the beta tubulin gene, TUB2 (tub2-443) as well as

microtubule depolymerizing agents such as benomyl. We reached to the conclusion that microtubules are not required for INM targeting of proteins.

INTRODUCTION

Inheritance of ER

During cell division, for most cells, the nuclear membrane and golgi vesicularize, and are reformed in each daughter cell after nuclear division. In contrast, the ER remains intact and partitions by, cortical ER moving into the daughter cells and pinching off by cytokinesis. Thus, during cell division, the daughter cell inherits about half the amount of the ER that is originally contained within the parent. This alleviates the requirement for de novo synthesis of ER.

In yeast, the ER tubules around the NM (perinuclear ER) and the peripheral ER close to the plasma membrane are spatially distinct. Yeast undergoes closed mitosis where the nuclear membrane does not disassemble as in case of higher eukaryotes. During cell division, as the bud grows from the mother cell, the nuclear membrane tends to elongate into the growing bud and is pinched off upon cytokinesis. Along with the nuclear membrane, the perinuclear ER that is continuous with the ONM is also inherited by the daughter cell. The cortical ER on the other hand is pressed against the plasma membrane and is inherited independently of the NM. Cortical and perinuclear ER have distinct mechanisms for inheritance (Fehrenbacher *et al.*, 2002). The cortical ER becomes attached to the emerging bud during early stages of cell division. As the bud grows, the cortical ER is pulled into the bud. This process requires the function of a type

V myosin motor Myo4p (Estrada *et al.*, 2003). Inheritance of ER is also affected by gene encoding products that play a role in ER-golgi trafficking (*SEC27, SEC21*) as well as targeting of ribosomes to the ER membrane (*SRP101, SRP102*) (Prinz *et al.*, 2000). Studies with various actin and microtubule depolymerizing drugs indicate that though the cytoskeleton is important in the inheritance as well as dynamics of the ER, they are not required to maintain cortical ER structure.

Tubules of the cortical ER do not extend towards the periphery in several mutants that affect binding of ribosome to the ER membrane. Slowing translation by use of sublethal doses of the protein synthesis inhibitor cycloheximide restores ribosome binding in mutants with a defect in the signal retention pathway (SRP) (Prinz *et al.*, 2000) and this in turn restores normal formation of cortical ER. Binding of ribosomes to ER seems to facilitate extension of these tubules towards the cell periphery. In support of this hypothesis, it has been observed that in yeast both cortical as well as perinuclear ER have ribosomes attached to the membrane and both seem to play an important role in protein sorting. Proteins that are involved in maintenance of ER structure may affect the targeting in an indirect manner by disrupting morphology of the ER tubules. This may be the cause of Trm1-II-GFP mislocalization in *ice2A*. This chapter discusses the studies conducted to understand how ER may be involved in protein targeting to the INM.

ICE2

YIL090W was discovered in a screen for genes that affect the inheritance of cortical ER (de Martin *et al*; 2005) and subsequently named *ICE2*. An ER marker Hmg1-

Figure 23: $ice2\Delta$ displays abnormal cortical ER formation. Indirect immunoflorescence of wild type and $ice2\Delta$ strains stained for an ER resident protein Kar2. Kar2 localization defines the ER tubules extending from the ONM in the wild type. In contrast, such extensions are not visible in $ice2\Delta$, thereby displaying a defect in the formation of ER.



GFP was used as a reporter in a screen to identify mutants defective in cortical ER formation. Hmg1-GFP displayed less peripheral staining, indicating a defect in the spread of ER tubules towards cell periphery (de Martin et al; 2005). Here I show that localization of the ER protein Kar2 by indirect immunoflorescence yielded similar results (Fig.23). The study by de martin *et al* (2005) also indicated that nearly 70% of the *ice2* Δ cells displayed a defect in inheritance of cortical ER into the bud. This is contrary to other mutants that exhibit defect in inheritance of cortical ER but do not necessary affect the ER network. For example, $sec3\Delta$ mutants exhibit severe defect in cortical ER inheritance but possess a wild type ER network which is absent in the *ice2A* strains (de Martin et al., 2005). de Martin et al (2005) also showed that Ice2 is detected in the cell lysates only when the membrane is solubilized with Triton-X-100 but not with high salt indicating that Ice2 is an integral membrane protein. de Martin et al (2005) propose that Ice2 is a type III integral membrane protein with the N-terminus of the protein is facing the ER lumen and the C-terminus is facing the cytosol. Additionally, they showed that though Ice2 is involved in inheritance and topology of cortical ER, the protein itself resides in the perinuclear as well as cortical ER. This is in concurrence to the localization data of the yeast GFP collection (Huh et al., 2003).

Previous studies indicate that the defect in abnormal cortical ER might be due to defects in binding of ribosomes to the ER (Prinz *et al*; 2001). This abnormality can be rescued by slowing down translation with cycloheximide so as to provide additional time for ribosomes to bind to the ER (Prinz *et al*; 2001). de Martin *et al* (2005) discovered that though *ice2* Δ strains exhibit a partial defect in the attachment of ribosomes to the ER

membranes, slowing of translation does not restore the topology of cortical ER tubules. Thus, the defect in the ER network observed in $ice2\Delta$ strains is the effect of some as yet unknown function of Ice2. In this chapter, I discuss our attempts to understand the physiological role of Ice2 within the yeast cell to provide clues as to how Ice2 functions in targeting Trm1-II-GFP to the INM.

KAR3

Kar3 was first identified in a mutant screen of *S.cerevisiae* for genes required for karyogamy (kar) or nuclear fusion after mating of haploid cells (Meluh and Rose, 1990). During mating, haploid cells of opposite mating types *MATa* and *MATa* respond to mating pheromone by arresting in G1 and reorganizing their cytoskeleton to create a projection known as shmoo. The two shmoo tips meet and fuse to create a zygote with two haploid nuclei in a common cytoplasm. In yeast cytoplasmic microtubules are dynamic only at the plus ends while the minus end is static and attached to the SPB. This facilitates shrinking of microtubules attached to the shmoo tip after zygote formation allowing the nuclei to oscillate towards each other (Maddox *et al*; 2000, Maddox, 2005) finally resulting in nuclear fusion. The cells lacking in Kar3 fail in nuclear migration and subsequent nuclear fusion or karyogamy, thereby creating a heterokaryon cell with two nuclei in a joint cytoplasm.

Subsequent studies have shown that Kar3 is one of 6 kinesin related proteins encoded by *S.cerevisiae*. *KAR3* encodes a kinesin-14 type motor protein with the motor domain at its carboxyl terminus. Kar3 forms heterodimeric complexes with kinesin associated proteins (KAP), Vik1 and Cik1. Cik1 is highly induced in response to mating pheromones and the formation of the Kar3Cik1 complex is essential for the process of mating, especially nuclear fusion (Barrett *et al.*, 2000, Sproul *et al.*, 2005). Cik1 is involved in targeting Kar3 to the tips of cytoplasmic microtubules during mating, facilitating depolymerization of the microtubules at the plus end (Sproul *et al.*, 2005).

In addition to its role in karyogamy Kar3 plays roles in various cellular functions including spindle assembly, microtubule depolymerization, chromosome segregation, spindle positioning etc (Meluh and Rose., 1990). These diverse activities are carried out by teaming with the Cik1 homologue, Vik1 (Barret *et al*; 2000, Manning *et al*; 1999). Though the Kar3Cik1 complex exists during vegetative growth, its presence is more pronounced during mating while Kar3Vik1 complex is formed in vegetative cell and has not been detected during mating. Vik1 and Cik1 play important roles in targeting Kar3 to various locations such as cytoplasmic microtubule ends, spindle pole body or mitotic spindles. *kar3*\Delta and *cik1*\Delta cells are sensitive to growth at 37° C, have long elongated cytoplasmic microtubules and short mitotic spindle. *vik1*\Delta strains in contrast are not sensitive to higher temperature and do not display any microtubule defects. However, they are resistant to microtubule depolymerizing drug benomyl (Barrett *et al.*, 2000).

ice2 Δ mislocalizes Trm1-II-GFP to the nucleoplasm. Our quest to understand how an ER protein involved in the inheritance of cortical ER affects targeting of a protein to the INM led us to *KAR3* because *ice3* Δ and *kar3* Δ are synthetically lethal. We predicted that probing the connection between Ice2 and Kar3 would help unearth the unknown physiological functions performed by Ice2.

RESULTS

ICE2 appears to be unique to fungi

Ice2 is an integral membrane protein encoded by the ORF YIL090W. Analyses of the protein sequence by various web based programs predict the presence of transmembrane domains (SGD, TMred). The different algorithms predict different numbers of transmembrane domains. However, upon close inspection of the protein we reached to the conclusion that the Ice2 has a total of 7 transmembrane domains. We reached this conclusion based on the stretch of amino acids between two consecutive transmembrane domains. Some of these consecutive regions had a very short non-membranous region (<5 amino acids) between which was not sufficiently long to form a loop between two transmembranous regions. This indicated that these two predicted domains may actually be a single transmembrane region.

Alignment of the protein by Dr. David Stanford encoded by the gene *ICE2* yielded surprising results. Ice2 appears to be restricted to fungi family. No protein with sufficient homology was detected in any of the other organisms. Also, within the fungi family, the transmembrane domains are conserved (Fig.24) while the inter-membrane regions are not. The distribution of Ice2 to the fungi family leads us to predict that it performs a function specific for this family.

Figure 24: Alignment of the predicted amino acid sequence of Ice2 and homologues of Ice2 identified in various members of the fungi family. Underlined sequences represent the predicted transmembrane domains

YIL090W	MTSLSKSFMQSGRICAACFYLLFDLLSIPISFKVGGLECGL	SFTVTLFTLYFITTTLNVLARRHGGRLYIFT	73
Sbay	MTSLSKSFMQSGRICAACF <mark>YL</mark> LFTLLSIPISFKVGGLECGL	SFTVTLFTLYFITTTLNVLARRNGGRLYIFLT	
Sklu	MLPRSLIQSIRgCLTTFYLLLVLITIPISFQVGGLYCGL	SFTVILFNLYFLTIILKIVSKKYN RLYYSIT	
Skud	MTSLSKSFMQSGRVCAACF <mark>YLLFTLLSIPISFKVGGLECGL</mark>	SFTVTLFTLYFITTTLNVLAKRHGGKLYIILT	
Smik	MTSLSKSFMOSGRICAACF <mark>YLLFILLSIPISFKVGGLECGL</mark>	SFTVTLFTLYFITTTLNVLARRYGGKLYIFLT	
Spar	MTSLSKSFMOSGRICAACF <mark>VL</mark> LFTLLSIPISFKVGGLECGL	SFTVTLFTLYFITTTLNVLARRHGGRLYIFLT	4
Sser	MSTMLSSLKRGFRIISGTS <mark>YLALILISIPISFKIGGLYCGL</mark>	SFTVTLFLLYFISTTLSIIARtNGNNFFIILT	1
Scas	MISSSFIRGIRICSATFVLFTLISIPISFKIGGLHCGL	AFTVSLFILYFLSTTLSIWARKYGSRIHILLT	
Sexi	MSLLME#FIRSVRICSGSF <mark>VL</mark> FILISIPIAFNIGGLYCGL	SFTVTLFNLYFISTTLHflakkwankfflfls	
Agos	MVPKGLLRSLRFALASFYLFLVLITVPISF0IGGLYCGL	SETVILEILYEVTITLKITAAtRGDE-WKHIA	70
Klac	MLHRITMEIFKQTRRRRSQERRLEPSTIARHCAKFNVSKMPLIKKSVLQTLRIFLTTMYLFLVLITIPISFQVGGLNCGL	SFTVTLFALYFVSTTfKLV1LDRGSALS	108
Kwal	MIPRSILQSIRVCLTSFYLLLILITIPIAFQVGGLYCGL	SFTVTLFNLYLITTTLKIAARaRG FAKLA	
Dhan	MNRI KVRTFRVLRAVLSTV <mark>YLALIILSIPLAFDVGGITCGL</mark>	AFSFItFTLYFVLITIRIVIRKSR YFGwV	70
Calb	MPIIKTITRSIRAILATLYIVLIILTIPLAFDVGGVNCGL	AYSLTtlVtYFILTTIRILvRKTK YFQwI	
Cgla	MGLITEGVQRSIR <mark>I</mark> SSAAL <mark>YI</mark> VL <mark>TLVSIPISFKIGGLQCGL</mark>	SFTVTLFNLYLISTTLSVLARRSCNRGYVFaT	73
Ylip	MITILTLFRAAYSLI <mark>HL</mark> TLVMITIPLSFDIGGRFCGL	AFSFILVVsYFILSIfRILSGRIKLRHVA	66
Ncra	MWFISASTSAI <mark>FL</mark> LS <mark>IVL1IPIAFDVGGRDAGL</mark>	AYSLSLFIFYLIYSTsKLLTPESSRVR-WFfT	64
Gzea	MWWLFKALFSSI <mark>FL</mark> LS <mark>IVLSIPVAFDVGGRDSGL</mark>	AYSLALFLFYFIYSSLELLTPEKSRSR-YFLS	65
Hjec	MWWPfSIFFSSVFLFAIVLSIPVAFDVGGRDSGL	AYSLGLSIFYIFYSaIRLATPETSRIR WTIV	
Anid	MwLFRILSSALFLTVTVsSIPLAFDVGGKTCGL	AFSLSLATFYFLFSVLKLATPKQSWFR-SSLI	64
Afla	FLSVTVs SiplafDVGGKTCGL	AFSLSLATFYFLFSLLKLTTPERSWLR ASLI	
Cpos	MWIVRLISSAFFLAIVLSIPLAFDVGGRTCGL	AYSLSLATFYFLLSVLKLTTPDNSRVR YALI	
Pbra	MWIFRIFSSALFLTAIVLSIPLAFDVGGRTCGL	gFSLSLAAFYFLFSLLRLTTPDCSWIR YSLI	
Cful	M ILRFIGSTLFLFSIVITIPLAFDVGGRTCGL	AFSLSLASYYFFLSILRIATPDESRWR KAaV	1
Spom	MDQEIRSNNYDISVSTVEDTSFEAMDDSILEIIDDEAPEIQGKPKANISTHFRNMLAMFGNFLSLGFFLVIIVL VgTAFEIGGRFCGL	ILTLALEVYFFSTALLKLfgLRKIA	113
Cneo	MGL LAVLAFTqglatfSqvLlGLPAaL	AL LopAS ILLSILYTahhLIYATLR LALK	
Fneo	MGI LAvLSFTqglat <mark>fS</mark> qvLLGLPAaL	SL LgpSSFLLLS1LfTahhL1YSTLR LCLK	
Ccin	MLNLWFIWSxDLFVSFERTDLRrLNRLStalQ <mark>ILIfLPLt</mark> La <mark>tLsRHAFL</mark>	1LSL1LSAhaLIHgTMALLwGsQALS VLQ	
Pchr	MAP WLIWKAX TEPSRITARRLStVIQILIfLPLtLstLatDAFL	1LSL1LFIhsLIHgTLTLLwGsPALS VLQ	
Umay	MSFVRALAIN GRVAtFI QVLFyLPLALDIAGQDAFL	ALSaSLASYYLFLSTVRLLTRaTRLAWIG	66
0.73			

				.						
YIL090W	NCLYYSQHFIIASLLYL	FLSGFSNDELGNVLK-	-NKYNESE	-SFLEALKN	SLNSNQINYVLYYYYR	F	VVQPWQ	FVLTKSTPFFT	LSEGFFTILA	IQAV 169
Sbay	SCLYYSQHFVIASLLFL	FLSGFSNDELGNVLK	NRSNESE	SFLEALKN	SLNSNQINYVLYYYYR	F	VVQPWQ	FILTKSTPFFT	LSEGFETILA	IQAV
Sklu	SFVYYLQHFIIPSLLFL	FLSGFNNDELKRQID	GDVNLPR	ESLVNFLRN	K SGKHSWIFYYYYK	F	TVKPWQ	FVLLHSTPYFT	LLEGFFTVLG	IQAI
Skud	NCLYYSQHFV1ASLLYL	FLSGFSNDELGNVLK	NRSNESE	SFLDALKD	SLNSNQINYVLYYYYR	F	VVQPWQ	FVLTKSTPFFT	LSEGFFTILA	IQAV
Smik	SCLYYSQHFIIASLLYL	FLSGFSNDELGNVLK	KRPNESE	SFLEALKN	SLNSNQINYVLYYYYFR	F	VVQPWQ	FILTKSTPFFT	LSEGFFTILA	IQAV
Spar	SCLYYSQHFIIASLLYL	FLSGFSNDELGNVLK	NKSNESE	SFLEALKN	SLNSNQINYVLYYYY	YR F	VVQPWQ	FVLTKSTPFFT	LSEGFFTILA	IQAV
Sser	SLLYYCQHSIIASLLYL	FLSGFSNiELHQLIEN	INSSLQLDSLT	SIFNGTSIGHNY	SNSIVNSNNLKANWIFYYFY	YK Y	IVVPWK	FILSHSTPFFT	LSEGFE	174
Scas	NILYYFQHFIIASLLYL	FLSGFSNEQLSSMLR	DGNKPID	SIADIFKK	NVASSTQANWVLYYYYR	F	FVQPWQ	FILSYSTPFFT	LSEGFFTILA	IQAI
Sexi	FILYYCQhLIIASLLY1	FLSGFSNND		DFQTMIRE	STKPTESNWIMYYYYK	F	FVKPWQ	ISLSFCSPFFT	MSEGFFTILA	IQAI
Agos	STLYYLQHFLMPSILFL	FLSGFNNDQLQKKLVG	SDPKGARGGE	IFDGNMNESLLQ	LLGRSRDDRATMAYLHYYR	Y	VVRPWQ	YMLLHATPCFT	LTEGFFTVLA	IQVI 176
Klac	TLIYYLQHLLIPSILFM	FLSYFeN			IDSDTVDSEYFKVFWN	V	MVKPWQ	LSLIH <mark>STPVFT</mark>	LLEGVETILA	IQAI 180
Kwal	SAaYYAQHFfIPSLLFL	FLSGFSNDELKHQID	TNTRPDE	SLIDLLRS	STHSQP WIFYYYYH	Y	VVRPWQ	FLLLRSTPYFT	LLEGFFTVLg	IQAV
Dhan	SLLYYLQHIIIPSLLTL	FLSYYrTN			QPTTLTAYHPH		EIWR	YFIVNSTPIFT	ILEGFCSLLL	IQAI 135
Calb	SIIYYCQHFfIPSLLTY	FLSYYSHENIKKLDL	ISS	SGSGSSTS	TSTSSKENP		VMIWR	LVLINSTPIFT	VLEGFCSLLL	IQAM
Cgla	SILYYSQHLVIApLLFL	FLSGFSNEEFNRFLSF	VGSYQELDPI	GSFLDLLKHRIL	MATTAENSKNWTLYYYYFK	F	IVQPWQ	WLLSYSTPFFT	LLEGFFTILA	IQAI 179
Ylip	QILYYLhtILIPALLIF	YLSLnTAN			NNE PVALQR		LVMPWD	ALMQS <mark>SaPFFn</mark>	ILEGFCTLLf	IQtI 131
Mgri								LSYSSPLFQ	pAEGFcTVpV	VQAA
Ncra	SSVgLSQWIIIPALLIW	SLNRFSVDAEN		AGWV SRTFS	SATANHKPSTWGEYFFGQHGI	EVENV	ALGAWD	KTLSY <mark>SSPVFQ</mark>	LLEGFCTLLV	IQtA 157
Gzea	GFLELSQWIIIPTLLIW	ALgQFAVDAGN		TNWVERTVG	-GLFNSKSTSWREWMFGKDGI	LIETV	TLGV <mark>W</mark> D	NVLRYSGPVFQ	LLEGFCTLLV	IQAA 157
Ebro		gqfavdaGS		INW GKIVG	gIFRSKSRswtew1VGRDGV	VLETV	MLGSWD	NTLRYAGPVFQ	LLEGFCTLLV	AAQI
Hjec	SLLRLAQWFVIPSLLIW	ALgKFAVDADA		GNSTWVERT IG	GVFHSRRTSWIEWMFGKDG	FLETI	MLGSWD	NVLRY <mark>SGPVFQ</mark>	LLEGFCTLLV	IQAA
Anid	VLISSLQWVIIPILLIW	SLNRFSVDTDNS		TSWVERTFS	GKRAQDSSIQDWLFGRDGI	LLESV	ALGNWD	KILRWSTPVFQ	LAEGFCSLLV	IQAA 156
Afla	TIIRSTQWIIIPVLLIW	SLNRFSIDADNT		TGWAERT FT	GKRAQNTSVQAWLFGPGGI	LVESV	TIGNWD	NLLr		
Cpos	ILaRsTQWIVaPILLIW	SLNKFSVDSDNS		SGWVERT FN	GKRAQDISIHEWIFGANG	LIETV	SLGSWD	KLLQWSTPVFQ	LAEGFCSLLV	AAQI
Pbra	AFIRSTQWLIVPIVLIW	SLNH <mark>FS</mark> VDAPDHHHST	TTTTTTTNLQG	RGIHZ						111
Cful	RTItgLQaFVIPTLLIW	SLNKFmVDGGtDAS		WVSRAFYN	IATKPLQHSPSLRDWVFGKDG1	LLESA	FIGGgD	KLLRYSTPVFh	laEGFCSLLV	IQAS
Spom	LTLHFFEpLLVfILLII	ALNAspSiEQ	NKYAS			F	LASAWN	TALLHITPLFn	LLEGMASLLV	VQAL 177
Cneo	ntPLaPLVsVA	AV1SpAIsAALA			LLTLYFYLAPPAPGATLAHO	CLVNI	LPFAYA	QLLRWVSPLFT	LLEGISTLLV	IQVA
Fneo	NTWLAPLVSVL	AVLSPAISAALA			LLTLYFHLAPPLPGPSLSHO	CLVNI	LPFAYA	QILRWVSPLFT	LLEGISTLLV	IQVA
Ccin	VPMHPFLLLLSFNVF:	SSSVNPWV					LTAVDWWG	KL <mark>LTLSGP</mark> LFi	aMEG1SSLLV	aQkL
Pchr	VPVHSFLLLVCFNAF.	AQTVHPWL					LTAAGWWG	WALRWSSPGFi	VMEGmSSLLV	VQkL
Umay	QTLaVFQFIVIPAcLLV	cfNVYSPpSATIFPPr	rpdsqdktps	eaisslfsrsha	tasldsldsqllqsltgfnsl	ldrivsasvAFF	WLARRVPGCWH	TLLRISSPLFS	LLEGVASLLV	IQSL 190
100 000 10 C	: : : *	:.	Contraction California California		and the second		*	: * *	: **. ::*	:*

YIL090W	GETNRWLSND	INSNTWI ISELLTSGGVITASLYVLYRIYVTPIWPLSIQTASLLGIVLSMVCGLGLYGIV 2	13
Sbay	GETNRWLSND	INSNIWI ISSLLASGGVITASLYYLYRIYVTPIWPLSIQTASLLGFVLSMVCGLGLYGIV	
Sklu	GETNKWLRYH	KNSNIWI IVSLLISGGVIITSLYYLYRIYVIPVWELSIQTASLLGFILSIVCGLGLYGIV	
Skud	GETNRWLSND	INSNIWI ISSLLASGOVITASLYYLYRIYVTPIWPLSIQTASLLGFVLSMVCGLGLYGIV	
Smik	GETNRWLSND	INSNIWI ISSLLASGGVITASLYYLYRIYVTPIWPLSIQTASLLGFVLSMVCGLGLYGIV	
Spar	GEINRWLSND	LNSNTWI ISSLLASGGVITASLYYLYRIYVTPIWPLSIQTASLLGFVLSMVCGLGLYGIV	
Scas	GEINKWLVYE	INSNTWV ISSLLASGGVITASLYYLYRIYVTPIWQLSVQTASLLGFTLSLVGGLGIYGIV	
Sexi	GETNKWLSNE	KKSNTWI ISSLLASGGVITTSLYYLYRIYVTPIWDLSIQTASLLGFVLSLVGsLGIYGIV	
Agos	GETGRWLRYK	RNSTAWV ISSLMVSGVVIITSMYYLYRIYVTPIWPLSVHTATLLGFTLSLVFGLGLYGII 2	14
Klac	GETERWLKMS	RR <mark>SNOWV ILSLLISGGIITLSLFYFYRIYVTPMWEL</mark> STPSASLLGFTFSIVCGIGLYGIN 2	25
Kwal	GEINRWLSRG	HGSNVWV ISGLMTSGGVITAALYVLYRIYVTPIWELSVQTASLLGFTFSIVSGLGIFGIV	
Pang		fp <mark>SLLGSAFTCTIL ITVFGI</mark> Y	
Dhan	GEIVNWLTVY	KSDSWL IVSIMASGSTITAALYFLYRIYVFP FTIDMISASLLGSLLTLTAGLGLFGIV 2	20
Calb	GQIMsWLTIY	KSDSWL IISLIGSGSVITASFYFLYRIYVIP FTIDMFSASLLGSILTTIMGLGLFGII	
Cgla	GEINKWLSYE	MNSNIWI IISLLVSGGVIIGSLYVLYRIYVIPIWKLIQSASLLGFVLSIVFCLGLYGII 2	14
Ylip	GQVSKWLVHR	KSDSWE IVLLISAGCVISATAYnLYRIYIat DSVDLSTgTLaGMVLTLgFcLSSYGVI 1	9
Mgri	GQMTRWLVNR	GRIDIWVEIVLIVISSSILISAAYFLWRIMKFP QINNIDATLIGVIIISAVFLGAYGIS	
Ncra	GRVARWLVTR	GGgD <mark>IWM¤I</mark> FLLAF <mark>SG</mark> SIISSAVYFLWRVAhFP EINTIDATLIGATMISAVFLGAYGIG 2	22
Gzea	GQLTRWLVNR	GRSDINLAIVLLVLSSStMASAVYFLWRVAQFP QIgNLDATLIGIAMTAVFLCAFGIG 2	2
Ebro	GQMTRWLVNR	GRSDTWVRIILLAFSGSIIASAV 1	0
Hjec	GQITRWLVNR	GRSDTWV IVLLAFSGSIIASAVYFLWRVAQFP QISNIDATLIGATMITAVFLCAYGIG 2	13
Anid	GQITRWLVNRG	GR <mark>SDSWMWIGLLVLSASVISSSVYFLWRVLOFP</mark> EISNVDAALIGVTVICAVILCAWGIG2	2
Afla		MIGLLVFSGSAISSAVYFLWRVLQFP GISNVDAALIGVSITCAVILCAWGIG	
Cpos	GQITRWEVNRG	GRSDSWMWISLIVMSASVISSSIYFLWRVLRFP EISSVDSALIGVAITCAVFLCAWGIG	
Cful	Ga hQYLVNQN	G lgyWM MAFM 1	7
Spom	GhLSRWLVHN	KSENWMATFILLNASSAISMSLYILYRVSS FSISNPNALMIGFSLATVIVISIYGVA 2	24
Cneo	GRVGKgwADEEDR	DEGLe <mark>WR</mark> tVVaLVAAALVyCAgLAgV VkTFP slPFpAfLLGAALTAVLFLSLIGFS	
Fneo	GRVGKgwADEEDR	DDGLewr tVVaLVAAALVyCAgLAgV IkVCPn 1PLpAfLLGAALTAVLFLSLIGFS	
Ccin	GQeGKkLIGVEQ	PKGEFYQ FALLIGTAVAFVASAYWIV DESYPSAAs <mark>spl5stllgvalt</mark> tflFLTVIGFV	
Pchr	GQVGKeLVEE	GEr <mark>YQ fALLIAAAAAYVTSAWWI</mark> V V <mark>ASYP</mark> SAAa <mark>S</mark> PMISTLLGVAVTAfIFLTAIGFA	
Umay	asTSRWIIAsslanpspryqhtsstatpsarGAAW	ISNTFIMRSLASIGFGA <mark>SEvNQ</mark> IFF <mark>LLVSATIYVTAAFALY</mark> vs <mark>F</mark> dgaT KerPGT <mark>AAaIatSVT</mark> STLWLTAIaFA 2	9
Aaeg		VNLSHRVPNLLSILTS <mark>A</mark> EfiQ <mark>LFFLLASAFVYVVSSLALY</mark> vs <mark>F</mark> EgaT RnrPG <mark>AAA</mark> a <mark>IGAAVS</mark> SALWLTAIaFA	
Mtru	AC137837.3	ISILFISLLetS	
	*:		

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YIL090W	SOKGSVIESSLE	FAYIVRCIYEISPKLATTATDEILNLFKDV	WOKHORNLPTAD-NLLC	YFHNWILKNAEVLWGSFIPRG	RKKTGDFHDKLISIL	333
Sbay	SORGSVIESSLE	FAYIVRCIYEISPKLATTATDEILDLFKDV	WOKHORNLPTAD NLLC	YFHNVILKNAEVLWGSFIPRR	RKTTSDLHEKIISIL	
Sklu	SCKGSVIESSLE	FAYIVRCIYEISPOLATSAMDEIFKIVKDT	WOKHOGNLPT D NLLS	YYRNVVLKNGEMIWDAIV	QKTG TTSPS	
Skud	SQKGSVIESSLF	FAYIVRCIYEISPKLATTATDEILDLFKDV	WOKHORNLPTAD NLLC	YFHNVVLKNAEMLWGSFIPRG	RKKTGDFHDKIISIL	
Smik	SQKGSVIESSLF	FAYIVRCIYEISPKLATTATDEILDLFKDV	WOKHORNLPTAD NLLC	YFHNVILKNAEMLWGSFIPRG	RKKTGDFHDKLISVL	
Spar	SQKGSVIESSLE	FAYIVRCIYEISPKLATTATDEILDLFKDV	WQKHQRNLPTAD NLLC	YFHNVILKNAEMLWGSFIPRG	RKKTGDFHDKLISIL	
Scas	TAKGSVIESSVE	FAYIVRCIYEISPKLATTATEEILEVFKEV	WQRHQANLtSPN NLLS	YYHNVVLKNIETINESF	KIND NSFITSF	
Sexi	SERGSVVESSLE	FAYIVRCIYELSPQLATSATDEIFAMFREV	WQARQNSfqIKD DLVF	YYGGnIvrNAQLL KSFKVK	lasp iylftff	
Agos	SGKGSaIESSLF	FAYMVhCIYEISPQLALTAMSEIFQVVHNT	WQMQQGTLP-KN-SLLA	YYKN-FILNAENL DVLLKKAFI	LHSVELDQAPSSVVVKDALVL	346
Klac	GSGSVMESSLF	FAYMVRNVYEISPKMAMNAMDEIFDIVKET	WKIHQANLPKKD-NLLE	LMTKNYRAI SQIVEH	TPRNHKYIKMM	334
Kwal	SGRGStIESSLF	FAYIVRCLYEISPQLATSAMDEISNLVKDT	WhNQHRSLrSSD TfLA	YYRDIILKNGEMINDAVLART	NVNVSGA GPV	
Pang	SGKaSPLECSLM	VAYIVKCcYELFPELSEMnFNmLLKFIMNEFK	K LdYQANSLnKEFLNsIQ	TTFNNfKTGrDLLDYVHLEFLA	KSTTK DKLLLVY	
Dhan	SGKGSMIESALL	FAYIVRCIYETFPILSEDASKtLTSIFTQTTF	N LRNEIPKLP PQItN	TVLQLLPFLAS		273
Calb	SGKGSMIESSLL	FAYIVKCIYETFPILSEAATKALANLFTLTTQ	N LKKEIPKIP PSILN	PISEVIPFIT		
Cgla	SRKGSVIESSLE	FAYIVRCIYEISPKLATRATEEILDLVKDV	WQTHQRNL1NRD-NLLA	YYHNVVLKNAEMVNESFILP	RSRSLNAM	335
Ylip	SRKGSslESSLI	1AYtVYCLYfIFrKsSTSePPvtIEYSS	KQQYPPLP PLIMD	SYSALAKS		260
Mgri	SGRGNPIESALL	FAYVVLCVYQIFTDYmPSdPAAAEALAAAQAA	qQPEFPPLP PFIMA	SWSTLRHMLA		
Ncra	SGRGNaVESSLL	FAYIVLCVYQIFTDYQPSAEAAAAAEQAAS	LQPDFPPLPPIIMA	SYSTLMHILS		291
Gzea	SGRGNPIESSLL	FAYVVLCIYQIFTDY1PSgNADqTHNQDNS-	EPDIPPLPPVIMA	SYSTIIHMLG		290
Anid	SGRGNPVESSLL	FAYVVLCIYQIFTDYQPShPVEqPVQSPSQ-	aGDFPPLPPIIMA	SYTTLMHALS		290
Afla	SGRGNPVESSLL	FAYIVLCIYQIFTDYQPS				
Cpos	SGRGNPVESSLL	FAYIVLCIYQIFTDYQPStPLEtPSVSSQ	PDFPPLP PIIMA	SYTTLMHTLS		
Spom	SGRaNLSEASLM	FLYIaYtVYMVcTDFgNPnTSSLEKPKFDY	LPPNILQ	XSVHYLISTIS		302
Cneo	1KRTNVLETSLV	FVYVVYSaWLSGvEaEMPSGTfgSSSSSFPPP:	S plRQSHP <mark>LP</mark> STVgP	SVNSLLSYaLHA		
Fneo	1KRTNVLETSLV	FVYVVYSaWLSGvEaEMPSGTfASSS WFPSP:	S plRQSHS <mark>LP PTV</mark> eP	SINSLLTYALHS		
Ccin	mRRTNIIESSgL	aVFIaYNVWLcdfDqASLpTRrhHV	AAARncD	SVQPLVVLrFLGN		
Pchr	mRRTNVIESSgL	aLvLaYNfWLcGfDqSPYwNPAAAZZVYRPPE	R eaSLTHLag	2HSAPLLSN		
Umay	iRKGNVIETSLM	FAYVVFNIYQLGPSLSFTpDPiSLIRSFKVNT	H gQAFVDKLP rS	LQAPLISK		363
Aaeg	VRKGNVVETALV	FAYVVWNVYQLSdSLSFTADP1ALIRSFKVLD	TStgTNLVK <mark>fP</mark> VLL	DNLPpIVY		
Mtru	TlfpSILESSIL	FftILYqsWLSGiE				
	:::	: * . :*	:	:		

Sser															174
Scas	RIMKVLYWYSPO	CILIAMYa	aHLILQY	5GELKD	ELCL	GCNSK	IFP S	DQPKIIVE	SWGFWN	WCNIF	FTILV	ASEL	IGTTPGSKQZ		486
Sexi	RFMRLLfWYSP(CVLIAMY1	THILL	SGESKd	DMCI	GCNSk	WVPTSFR	KPQDe iVV	/Nsg <mark>FW</mark>	WCHIF	Wallvi	(SyEL	IGTCTIQRZ		479
Agos	KLMRI I YWYSPO	CIVIAMY	THMILRY	5GELNN	ELCI	GCFPW	LQSEA	NRDRWVVI	AWG FWN	WCNLF	FTLLI	AVEL	IGSR		502
Klac	RIMnFVYWYSP(CIIIAMY	THLILRY	5GQLKN	DLCI	GC	FPYE	TEPKIVV	SWS FWI	WCNIY	STMLI	ALEL	MGSNK		486
Kwal	RFMRMLYWYSPO	CIVIAMY	THLIMQY	SGQISN	DLCL	GCFPW	SPP	EGSKVVVE)SWG <mark>FWI</mark>	WCNIF	CTMAI	ASEL	FGSKSGZ		475
Pang	tsSRL <mark>IY</mark> 1YSP(CIIIAVY	TNLMIQY	IdELDT	HNHF	NWLNFQ	FGGSSAQV	PEVInsVH	ISWQv <mark>WI</mark>	WINIE	V				280
Dhan	RL <mark>IY</mark> SYSP(CIVIAVY	THLMM1 YI	KGELGT	ELKI	GWWS	PN	SDIQ <mark>VIV</mark> H	Ip <mark>WQ</mark> FWI	NWVNMG	TLLL	(tA <mark>EL</mark>	LGNDSNSGRT	ALTSHNKVE	494
Calb	RL <mark>IY</mark> AYSPO	CLIIAVY	THLMLsYN	NGELGT	ELKL	PLWNSSI	GGGGGD	DDIQ <mark>LIV</mark> H	Ip <mark>WQ</mark> FWI	WINMG	TLIL	AAEL	SSNDNSSGGG	ISLTSHNKVE	494
Cgla	RILKLLYWYSPO	CVLIAMY	THLILQY	SGELKR	ELCL	GCTSN	WFGSK	DQPRIVI)SWS <mark>FW</mark>	WCNI F	WTVLV1	ANEL	IGTQAPNELT		499
Ylip	RrVFILYAYAP(CVIIAVY	THLIMQNI	dILHVPQ	NSGM	DWS		AÇ	QGsQ <mark>lW</mark> a	a <mark>WVgV</mark> 1	Af <mark>LAL</mark>	(AV <mark>EL</mark>	SsGGNMQNE	FIRDHWKEE	404
Mgri	KLLSVLSWFSP3	SILIAVY	TSLLLQHI	TVAADS	DIG V	TLT		AGDI	.GGNp <mark>W</mark> F	R <mark>WVNV</mark> G	ATMLM1	(SLEL	YLGDDEPAA	HWKSDZ	305
Ncra	RLVGFLSWFSP:	SILIAVY	TSLLLQHI	SVSNNGD	DIG V	TLR		AGDA	AGGNpWF	R <mark>wvnv</mark> a	ATMgL1	AVEL	YLGERNEGM	HWRAD	437
Gzea	KFLGFLSWFSP:	S <mark>ILIAVY</mark>	TSLLLQHI	STSDGP	DG	TLR		gGDV	/eGSn <mark>W</mark> Ç	Q <mark>WaNI</mark> G	LTMVL	GVEL	YLGS DEHD	HWKVD	432
Ebro															104
Hjec	mFLSLLSYFSP1	TILISVY	TS <mark>LLLQ</mark> HI	STSEGP	DGI	TLR		gGDV	/GGSt <mark>W</mark> F	RWINVG	LTMVL	GVEL	MLSTDDQ		315
Anid	QFLGFLSYFSP:	SILISVY	TSLLMQHI	ATTSQAMO	GSGQ	WST		qGAG	GGN <mark>1W</mark> F	RWINLA	GTLAL	AIEL	WLGAADDLDS	GLAGHWKTD	443
Afla															206
Cpos	QFLGFLSWFSP:	SILIAVYI	TSLLMQHI	AAQSTGA	N GE	WKS		AGAM	(GGN <mark>FW</mark> F	R <mark>W</mark> VNLG	CTMgL1	AVEL	WLGKQDDIDG	GLTGHWKTDZ	427
Pbra															111
Cful															178
Spom	K qGMwSilSP	CILIAVY	TNLLLQH1	LYPTPS	F	TSP	V	N q <mark>IL</mark> Q	:SaE <mark>iW</mark> F	RWVSai	LTLLL	AIEL	AySKESDIGQ	ALASHFKLD	441
Cneo	RITTVfLSYRR/	AILIAVYI	THLL <mark>x</mark> L					LDGG	SQTWWF	RWVNIA	LILSV	VSLEL	LLdAEGDDT	DSVTrWKVD	407
Fneo	RITTVfLSYRR	AILIAVYI	THLL <mark>x</mark> L					LDGG	SQT <mark>WW</mark> F	RWVNIA	LILAV	VSLEL	LLdAEGDDT	DSVTrWKVDZ	406
Ccin	tLTRLLLT <mark>Y</mark> KQ1	fIfVtVYS	SHLL <mark>x</mark> L					LDHS	SQI <mark>WW</mark> F	R <mark>WINI</mark> E	FTLfI	VSIEL	VVSSDD	EVSKeWKIDZ	385
Pchr	RLTRLLLTYRQ/	AlfVtaYS	SHLLXL					LDHE	PAQV <mark>WW</mark> F	R <mark>WMNV</mark> E	FTLIL	VAVEL	LVGNDDD	PVAKt WKVEZ	382
Umay	IISI <mark>LV</mark> S <mark>YS</mark> RI	1 <mark>ILIAVY</mark> S	SHLLL					LDQA	ANQI <mark>YW</mark> F	RFL <mark>tV</mark> G	LTLVL	GLEI	TISKEDDVGV	GVGD1gaAAAKAZ	521
Aaeg															237
	*		*									•			

T.

Т

YIL090W Sbay Sklu Skud Smik

Spar

Т

RIMKGLYWYSPCILIAMYTHLILQYSGELKK RIMKGLYWYSPCILIAMYTHLILQYSGELKK RIMRLYWYSPCILIAMYTHLILQYSGELKK RIMRGLYWYSPCILIAMYTHLILQYSGELKK RIMKGLYWYSPCILIAMYTHLILQYSGELKK RIMKGLYWYSPCILIAMYTHLILQYSGELKK

1

DLCINGCSEK DLCINGCSEK DLCLNGCFPW DLCINGCSEK DLCINGCSEK DLCINGCSEK

YIL090W	SFEKVSLISKPF KÆFKN TFS <mark>VPL</mark> SINEFCQVTIKMASESVSPAIVINLC <mark>FRVLMFYSATRIIPALQ</mark> RK-NDKQLRKSR 412
Sbay	SFEKVSLISKPF RECKN TFSVPLSINEFCQVTIKMASESVSPAIVINLCFRVLMFYSATRIIPALQRK NDKQLRKSR
Sklu	TTQMVWLRLQPL <mark>KKTKN TISVPS</mark> SIREIFRVTLKMATESVTPTVVINLV <mark>FRILVFY</mark> SATRIIPALQKD NPNGRKSSR
Skud	SFEKVSLISRPF <mark>MKFKN</mark> TF <mark>SVPLS</mark> INEFCQVTIKMASESVSPAIVINLC <mark>FRVLMFYSATRIIPALQ</mark> RKNDKQLRKSR
Smik	SFEKVSLISKPF <mark>KKFKNTFSVPLSI</mark> NEFCQVTIKMASESVSPAIVVNLCFRVLMFYSATRIIPALQRKNDKQLRKSR
Spar	SFEKVSLISKPF.K.FKN TFSVPLSINEFCQVTIKMASESVSPAIVINICFRVLMFYSATRIIPALQRK NDKQLRKSR
Scas	STERVITIFMPI KILKT TITVPSSINELCFVTYKMACESISPAVIVNLCFRVLIFYSATRIIPALQRR DDNELRKSR
Sexi	sltninIVSQPI N FKN TVTVPS <mark>S</mark> ITQLFWIVFDMASESVSPAVVINLCFRILIFYSATRIIPALQRK nSRERRSR
Agos	VQQQWWNKSQQL'S TKN SAgIPRSIHEIFIVTLGLARESINAAVV3NLVFRVIVFYSATRIIPALQRATGgTQSR423
Klac	DQCKWILNPL NSVKN TKSVFISIQEIFVVTFKMAKESLIPAVVINIVFRLLIFYSATRIIPTLQRNTKERRSfR 410
Kwal	AWIS WHRVOPL K VKH TSSVESSIQEIFLLTLSMAREAIAPAVVINLCFRILIFYSATRIIPALORK HK RRSR
Pang	VKVKEFIVDIVIkFVTEN PKSfe SIWDF LKISISNLTISILIQIAYRICVFFAATKIIFILQ V QKCSpLSTPSSPTPVKANRHETRVRNKS
Dhan	NLPgSFKAIWE LIMSINkLPIPLLLNIAYRIGVFYAATKIIPSLyhD ASYPINTPP RTPsqnrsprpsstnipg 348
Dhan	lsqsvsnsqtppnshhnrthetsseevidtteiqplsrsqssfarn-sfgkkreprstii 407
Calb	STLPSSFKGV.K
Calb	LR _{tk} NSFRLKPPhHQPPS VII
Cgla	EKIRFWLDVTPV K FKN TVSVESSIVELVSVTYKMASESLSPAVIVNLCFRILIFYSATRIIPALORS-SYKEQRQSR 414
Ylip	TAAI <mark>VP</mark> SSFTAVFDFFYgAISTITPHVfVSLSFRIGVFYAATRIIPgIh _V A GGFTTRYFRSY 322
Mgri	SLP eAFNVSFTFLYAAFQTVTFSVIISLAYRITVFYCATRIIPAVRESGTNALMADPELYGDSSDGAT
Ncra	SLP-eAFNTSFQFLYAAFQTITPSVIISLTYRIIVFYCATRIIPAVREL GARALMDEPSLLEETDGAN 358
Gzea -	SIP-havhsslällya <mark>a</mark> fQ <mark>TITPSVIISLTYRsLVFYCATRIIPSIRE</mark> S GAQAMMQEPD-WEDSETAS 356
Hjec	ARGAMLHEPD WDESEAAS
Anid	ILP-SIIHAAFNMITAVFSAVTPSVLISLTYRILVLYASTRIIPAVREA GARALSQEAS LDDSDAAG
Cpos	ALP SIIHTAFNIVSAAFSAISPSVLISLLYRLFVLYASTRIIPAVRES GARALSQEAS LDDSDGAG
Spom	ATLP-rtLYNIVLFMVAAAKTVAPSVfaTfAFRISVMYAVTRILPAIQNN IIFLEYSRTS 361
Cneo	AVP SFI UVSETLPPHLVLSLIYRVTVLH1AAKIVPvLRRA SMGWDDRDE REGFWDGRTLGDEPARNM
Fneo	AVP SFI KVSETLPPHLVLSLIYRVTVLH1AAKIVPvLRRA SMGWDDRDDAREGFWDGRTLGDEPARNM
Ccin	1VP HLQTLL nVInTLAKPVLIALLYRLTILHSASRILPTIGAD SWEGDGDEDN EWDRRP MTS
Pchr	ilp HLQTLM NfITyTLPKPVLVALLYRLTVLHfASRILPTIgaD AWEGDTDSDE GWDGRP MTS
Umay	ilraaSgllq <mark>y</mark> iAhWlGHSVDFLHA <mark>A</mark> GA <mark>ALFKSVIVSLIYRLMVLYAASRILFmLK</mark> gS E ELEEEVWDEDEG RWILQRRRLRRRRRKTFE
Aaeg	M1VdVaSsALrqiArFA AAAAALPKSVIVSIVYRLMVLYCASRILP1LtgNTAKPWDGETsS WGSVIGSESDSSEDSNDSHDDEKHISRTRKQNGKG 237

WFG VDQPE<mark>IIVDSWGFWNWCNIFCTILVYATEL</mark>IGSGS WFG PDQPEIIVDSWGFWNWCNIFCTILVYATELVGSGSZ SHTNDPSSKVVVDANGFWNWCNIFCTILYYATELIGGSSZ WFG AEQPEIIVDSWGFWNWCNIFCTILVYATELIGSGSZ WFG SDQPEIIVDSWGFWNWCNIFCTILVYATELIGSGSZ

491

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ICE2 and *KAR3* function in similar cellular pathways

A genomic synthetic lethality screen, using $kar3\Delta$ as the query strain, by Tong *et al* (2002) uncovered *ICE2* as synthetically lethal to *KAR3*; i.e. each mutation when alone is not lethal but the combination is lethal.

Synthetic lethal interactions between the two genes usually result by one of the three mechanisms. (1) The genes have redundant functions in an essential pathway such that mutation of both results in disruption of the pathway; (2) each gene performs distinct steps in the same pathway which though highly decreased, still retains function but ceases to function when two steps are eliminated; (3) the two genes interact indirectly, maybe by affecting a common pathway. The fact that Kar3 and Ice2 reside in different cellular compartments, which they do not possess any sequence homology and that *kar3* Δ does not mislocalize Trm1-II-GFP (data not shown) favors the second or third possibility for interaction between these two gene products. This would indicate that *kar3* Δ and *ice2* Δ might have phenotypic similarities.

One of the striking phenotypes of $kar3\Delta$ is the presence of highly elongated cytoplasmic microtubules due to lack of plus end depolymerization (Saunders *et al.*, 1997, Endow *et al.*, 1994). To determine if *ice2* Δ shares any of these phenotypes we performed immunoflorescence to study the length of microtubules in *ice2* Δ strains. As anticipated we found that *ice2* Δ cells display abnormally long cytoplasmic microtubules (Fig.24). However, as *kar3* Δ does not cause Trm1-II-GFP mislocalization, the length of the microtubules is not responsible for mislocalization of Trm1-II-GFP in *ice2* Δ (data not shown). A reason for elongated microtubules may be the absence of Kar3 at microtubule **Figure 25**: *ice2* Δ displays defect in microtubule depolymerization. Indirect immunoflorescence of to detect the beta-tubulin, Tub2 in wild type, *kar3* Δ , *ice2* Δ , indicates the presence of elongated cytoplasmic microtubules similar to that seen in *kar3* Δ .



Anti-Tub2p

ends and this may happen if Kar3 is mislocalized in *ice2A* strains and it would be interesting to learn if this is the case. Likewise, there may be a general defect of *ice2A* cells in targeting proteins to the INM, including the SPB and this may provide an explanation as to why Trm1-II fails to associate with the INM in *ice2A* mutants.

Microtubules do not affect localization of Trm1-II-GFP

Microtubules play an important role in movement of cargo to different sub-cellular locations. In higher eukaryotes, microtubules play an important role in dynamics of the ER tubules within the cell. However, in yeast, this has shown not be true (Prinz *et al*; 2001). A recent report indicating that proteins with NLS tend to be translocated along microtubules (Ems-McClung *et al.*, 2004) aroused our curiosity to study the role of microtubules in targeting Trm1-II-GFP to the INM. We used two different methods to analyze microtubules for this purpose, a temperature sensitive mutant (a gift from Dr. Charles Boone) in β -tubulin, *tub2-443* and a microtubule depolymerizing drug, benomyl.

To study the effect of mutations in essential genes such as *tub2-443* on targeting Trm1-II-GFP to the INM, it is critical to control the expression of Trm1-II-GFP so that the protein is expressed only after the essential gene product has been destroyed. In case of constitutive expression of the protein, there would be enough wild type protein already present at the INM and disruption of any essential function may not have much of an effect if Trm1-II-GFP does not have a high turnover rate. So we constructed a vector expressing Trm1-II-GFP under control of Gal1/10 promoter. This galactose induced

Figure 26: *tub2-443* does not affect the INM localization of Trm1-II-GFP. (A) *tub2-443* does not grow well at non-restrictive temperatures of $>34^{\circ}C$ (B) Indirect immunoflorescence with anti-GFP antibodies do not show a difference in Trm1-II-GFP localization in *tub2-443* cells at permissive and non-permissive temperatures. (C) Immunoflorescence using antibodies against Tub2 shows that microtubules are not depolymerized in this mutant.





protein displays wild type nuclear rim localization. Therefore, we opted to utilize this construct for our microtubule studies.

tub2-443 strains were first tested for temperature sensitivity (Fig.26A) and their effect on Trm1-II-GFP targeting. Defects in tubulin did not have any apparent effects on the localization of Trm1-II-GFP (Fig.26B). Indirect immunoflorescence was carried out to confirm the effect of higher temperature on microtubules. The mutation *tub2-443* does not seem to affect its polymerization; rather it affects the depolymerization of tubulin which results in the presence of stable, elongated microtubule filaments (Fig.26C).

Benomyl affects Galactose induction of Trm1-II-GFP

The wild type yeast cells were treated with microtubule depolymerizing drug benomyl to study the effect of microtubule deplymerization on Trm1-II-GFP targeting. DMSO treated cells were used as control. The protein was induced by addition of galactose in both benomyl and DMSO treated cells. Indirect immunoflorescence revealed the presence of shortened microtubules in cells treated with benomyl but not DMSO treated ones (Fig.27A). However, in cells treated with the drug, we could not detect any induction of Trm1-II-GFP (Fig.27B). This effect may be the result of a defect in translation, transcription or just quenching of the GFP signal by the drug. Further studies need to be conducted to explore these possibilities.
Figure 27: Benomyl inhibits the galactose induction of Trm1-II-GFP. (A) Indirect immunoflorescence with primary anti-Tub2 and CY3 tagged secondary antibodies indicates benomyl treatment depolymerizes microtubule as exhibited by shortened tubules. Control cells treated with DMSO alone do not show a decrease in microtubule length. (B) DMSO treated control cells exhitbit nuclear rim localization of Trm1-II-GFP (left) whereas, benomyl treated cells do not exhibit any induction of the fusion protein.







DISCUSSION

In this chapter we have discussed the studies conducted to understand the role of Ice2 within a yeast cell. Ice2 is a protein that localizes to perinuclear and cortical ER, but it plays a role in formation of cortical ER. Inefficient targeting of Trm1-II-GFP to the INM indicates that Ice2 probably plays a role in targeting of proteins. However, this could be a secondary effect seen due to inefficiency of the nuclear tether of Trm1 to be targeted to the INM in *ice2A* strains. Ice2 has only recently been implicated to have a role in ER inheritance. Thus, information regarding its physiological roles is scarce.

Our first attempt towards understanding the role of Ice2 was to examine its sequence. We hoped to find homologues of the protein in other organisms, thereby providing clues as to its function. However, protein homologues of Ice2 do not seem to exist outside the fungi family. Another interesting point is the fact that the predicted transmembrane domains in this protein are conserved across the fungal species. Unfortunately, the homologues in other fungal species have not been studied, so we could not draw parallels. The uniqueness of this protein in the fungal family indicates that this protein probably performs a function that is required in this family but not necessary or absent in other eukaryotic species. The major difference between fungi and the rest of the other higher eukaryotic families is the disassembly of the NE during cell division in the latter and absence of the same in the former. During cell division, the NM is reformed around each daughter cell. The occurrence of closed mitosis and presence of the NM during cell budding in yeast and other fungal species necessitates independent

mechanisms for the inheritance of cortical and perinuclear ER. Since this constraint does not exist in the other organisms, it seems more probable that genes involved in inheritance of cortical ER could be exclusive to the fungi family. This could explain the sequence conservation of this protein within yeast and its family members and its absence in higher eukaryotes.

Many of the genes whose products function in maintenance of cortical ER, such as *SEC27* and *SEC21*, are essential for cell viability, but *ICE2* is not. So maybe the abnormality in cortical ER observed in *ice2* Δ strain could be a secondary effect due to the inefficiency of some of these essential proteins to perform properly. Also, ER tubules are not synthesized de novo in the daughter cells but are rather inherited from the mother. A defect in inheritance of ER may affect the viability of the cell. It is possible that this defect has an effect on the longevity of *ice2* Δ strains and it would be interesting to test this possibility. It would also be interesting to learn if temperature sensitive mutants of *SEC21* and *SEC27* affect Trm1-II-GFP localization to the INM; at higher temperatures when the cortical ER is disrupted. If so, then it would indicate the importance of the cortical ER structure in targeting Trm1-II-GFP to the INM, if not, some as yet unknown function of Ice2 is responsible for the mislocalization of Trm1-II-GFP.

Tong *et al* (2002) in a recent screen found $kar3\Delta$ to be synthetically lethal with *ice2* Δ . Kar3 is a well studied protein and its diverse functions though still under scrutiny have been largely deciphered. Genetic interaction between *ICE2* and *KAR3*, provides an exciting endeavor, due to precedence for the interaction of SPB components with peripheral INM proteins. A recent study by Niepel *et al* (2006) shows that SPB

components Spc42, Spc110 and Spc29 co-immunoprecipitate with Mlp2, a peripheral INM protein and the SPB components mislocalize in $mlp2\Delta$ cells. Thus, *ice2* Δ may affect localization of Trm1-II-GFP indirectly by mislocalizing a SPB component. Therefore, we attempted to understand the physiological role for Ice2 via Kar3.

After, confirming the synthetic lethal interaction between these two genes, we searched for the phenotypic similarities. A characteristic of $kar_{3}\Delta$ is the presence of elongated and stable microtubules in vegetative cells. Surprisingly, *ice*₂ Δ exhibited the same phenotype. We have yet to test the other phenotypes such as nuclear fusion defect, chromosomal instability due to defects in mitotic spindle assembly, etc. Since $kar_{3}\Delta$ does not affect the INM localization of Trm1-II-GFP, elongated microtubules are not the cause of mislocalization of the protein. It is known that resident proteins of the ER are involved in protein targeting and it is possible that Kar₃ is not efficiently targeted to its subcellular locations in the absence of Ice₂. So, it would also be interesting to see if *ice*₂ Δ causes mislocalization of the Kar₃. It would also be interesting to see if *kar₃* similar to *ice*₂ Δ displays a defect in inheritance of cortical ER. This can be easily visualized by use of florescent tagged ER residing proteins such as Kar₂ or Hmg1. Ice₂ and Kar₃ interact in an as yet unknown manner. Understanding this interaction will lead towards a better appreciation for the role of Ice₂ in INM targeting of proteins.

The next approach we adopted was to examine the role of cytoskeletal components in targeting proteins. The fact that microtubules and actin play a role in targeting of organelles and proteins to various cellular locations as well as the fact that actin is important for ER dynamics led us to examine the effect of these cytoskeletal structures in targeting of Trm1-II-GFP to the INM. Actin and microtubule have been shown not to affect the structure of cortical ER. The tubulin mutation *tub2-443* we used for our study stabilized microtubules and we were interested in analyzing the effect of microtubule depolymerization. We therefore employed a depolymerizing drug, benomyl. Surprising, upon addition of this drug, we could not detect any GFP signal from the fusion protein. It is possible that benomyl affects galactose induction of the protein. This effect may be at the stage of transcription or translation. Further studies are required to eliminate any of the possibilities. Overall, we conclude that microtubules are not involved in the targeting of Trm1-II-GFP. We need to determine the consequences of the disruption of actin to assess the role of the actin cytoskeleton in targeting of proteins to the INM.

Finally, based on our studies, we propose a model (Fig.28) to explain the mislocalization of Trm1-II-GFP in *ice2* Δ . We propose an indirect role for Ice2 in INM targeting of Trm1. Trm1-II-GFP is inefficiently tethered to the INM in *ice2* Δ causing accumulation of the protein in the nucleoplasm. This may indicate the absence of sufficient copies of the nuclear tether of Trm1 (NTT) in these mutants. This is not surprising as ER resident proteins are known to function in protein targeting. Formation of abnormal ER may result inefficient targeting of NTT to the INM, which in turn may affect the tethering of Trm1-II-GFP causing it to mislocalize.

Figure 28: Cortical ER proteins play an important role in targeting proteins to the INM. In *ice2* Δ , abnormal cortical ER, results in inefficiency in the targeting of the membrane tether of Trm1 to the INM. This results in deficient localization of Trm1-II-GFP to the INM, and its mislocalization in the cytoplasm



General Discussion

A cell, prokaryotic or eukaryotic can be considered as a self sustaining site of a large variety of macromolecules. These macromolecules perform various different functions. DNA, a macromolecule composed of sugar, phosphate and nucleosides, is responsible for retaining and storing information required for the functioning of a cell and passing down this information to posterity. Proteins, composed of amino acids, are responsible for carrying out the all the functions as per the instructions contained in the genetic material, DNA. RNA forms communication link between the DNA and RNA. Other macromolecules within the cell include lipids that form the membranes. The lipids aid in discerning sub-cellular compartments or organelles, each of which perform specific functions.

The working of a cell can be considered to be analogous to that of a company, DNA being the CEO, RNA the managerial staff and proteins the workers. Each of these macromolecules are essential for the viability of the cell, however, the minute by minute chores are carried out by the workforce, the proteins. The proteome of the cell carries out a diverse array of functions, including, respiratory activities to produce energy, synthesis of RNA, replication of DNA and cell division, synthesis of proteins so on and so forth. Each of these functions if observed closely would require an individual protein to interact with a plethora of other molecules, these may include, other proteins, DNA, RNA or membrane lipids. The genome of a unicellular organism such as budding yeast encodes for around 6000 proteins. These, carry out the diverse range of activities of a cell. The sequence of a protein not only determines the function it carries out but also the macromolecules it interacts with in the cell. For example, the DNA binding proteins, which may function in DNA repair, gene silencing, DNA replication. These proteins will have a catalytic region determining their activity, and a region for binding DNA or the DNA binding domain. Similarly, RNA binding proteins generally possess RNA binding domains. Protein-protein interactions are considerably more complex. These may involve recognition of structure, charge (determined by amino acid composition), post-translational or co-translational modifications.

Modifications and protein targeting

More than 300 different modifications are found to occur on proteins. These include phosphorylation, lipidation, nitrosylation, glycosylation, methylation, ribosylation, acetylation, sumoylation, ubiquitination and many others. Modifications change the size, charge, structure and conformation of proteins. As a result, characteristics of the protein such as binding affinity, enzymatic activity, hydrophobicity are altered. It is therefore not surprising that cells have developed a method of controlling various activities or functions of a protein by modifications. Histones are probably best characterized for differences in function exhibited by different modifications. Histones may be acetylated or methylated. Methylation of histones is associated with silencing of chromatin whereas the more actively transcribed regions are often associated with

acetylated histones. Similarly, there exist numerous instances of phosphorylation controlling the activity of a protein. Not only the type of modification but also the site of modification plays an important role in determining the function of the additional group. The best example for such a modification is probably ubiquitination. Proteins may be modified by addition of a single ubitquitin molecule, monoubiquitin or a long chain of polyubiquitin. A polyubiquitin chain is formed when ubiquitin is attached to a lysine within ubiquitin itself, and this process is repeated. When the polyubiquitin chain is linked through Lys-48, it acts targets the protein to the proteosome to be degraded. Whereas linkage of ubiquitin through other Lys-63 is important for its role in DNA repair (Pickart 2001). Modification of proteins via monoubiquitination has many diverse roles. Monoubiquitination of the histories H2A and H2B at the carboxyterminal ends plays an important, albeit undefined role. Yeast harboring a mutant H2B that lacks the site of ubiquitination, grows much slower than the wild type and is defective in sporulation. Thereby, indicating that H2B ubiquitination may play a role in meiosis. Similarly, monoubiquitination has been reported to be important for endocytosis, viral budding, and regulation of DNA repair and so on (Hicke 2001).

One such protein modification that has perplexed the scientific community for long is N-terminal acetylation of proteins. It seems to have diverse functions as mutants defective in N-terminal acetylation, namely *ard1*, *nat3* and *mak3*, display a variety of defects in yeast. These defects range from growth; sporulation, silencing, and DNA repair encompass most of the major regulatory functions essential for cell survival. Recent studies have deciphered some of the roles for this N-terminal modification. Two proteins involved in heterochromatin silencing, Sir3 and Orc1 have been shown to be Nacetylated by NatA (Ard1 and Nat1) complex (Geissenhöner *et al*; 2004). It has been proposed that lack of this modification disrupts the interaction of Sir3 and Orc1 with other proteins involved in silencing and thus leads to derepression of heterochromatin. Nacetylation of Sir3 and Orc1 present an instance of how lack or inefficient interaction between proteins due to absence of modification can cause defective silencing of chromatin. Disruption of other protein-protein interactions can cause mislocalization of protein.

When we embarked on the project described in this thesis, our aim was to detect the interactions responsible for sorting peripheral proteins to the INM. In this thesis, I have described how our investigation led to the discovery of four genes, previously unimaginable to be involved in this process. Three of these genes encode components of N-terminal acetyltransferase complex NatC, for their role in targeting peripheral proteins to the INM. In contrast to NatA, NatC substrates seem to be part of the class of proteins expressed at low levels. So there are not many known physiological substrates of NatC. The proteins identified to be NatC substrates include, Pre5, Pup2 (proteosomal subunits), *gag* proteins of L-A virus, Arl3 (a small GTPase in golgi) and Trm1 discovered in our study described here. Many of these some NatC substrates either are or may potentially be associated with an organellar membrane. N-terminal acetylation of Arl3, an ARF related small GTPase is essential for the targeting this protein to the golgi membrane where Arl3 interacts with an integral membrane protein Sys1 (Setty *et al*; 2004, Behnia *et al*; 2004). Lack of N-terminal acetylation of gag causes defect in viral envelope assembly (Ribas and Wickner; 1998). Viral assembly is mediated as N-terminal Gag domain primes capsid assembly by homologous interaction with free Gag. Thus, the N-terminal acetylation is interaction with other Gag molecules. The proteosomal subunits Pre5 and Pup2 are nucleoplasmic. Electron micrographs have revealed that they are close to the NM. Thus, the N-terminal acetylation of Pre5 and Pup2 may be involved in either the interaction with other subunits of the proteosome or the NM. Finally, our reporter, Trm1 exhibits a rim like localization pattern around the NM. Trm1 may be peripherally associated with the NM via interaction with an integral membrane protein.

A recent report about NatB substrates indicates that many of these proteins are expressed at lower levels and most of them function in DNA repair, cell cycle progression and DNA recombination (Caesar *et al.*, 2006). Comparison of the codon adaptation index (CAI) of putative NatB substrates with that of all proteins revealed that the NatB substrates have a much lower CAI index and are probably expressed at a low level.

A cursory examination of the N-terminal protein sequences to identify potential NatC substrates indicate, a vast majority of these proteins are targeted to organellar membranes such as mitochondria, ER, golgi or nucleus. The entire proteome data available at Saccharomyces genome database (SGD) was sorted based on the amino acid sequence and the localization of each protein was manually determined using the information on SGD. This may mean that peripheral membrane proteins need a structural feature that allows them to interact with an integral membrane protein. The similarity of the N-terminal sequence, namely N-acetylation of methionine and the presence of hydrophobic amino acids may provide such a structure to aid in the interaction and tether of peripheral proteins to the membranes of organelles. This would require a more wide scale study to confirm the identity of NatC substrates and the importance of N-terminal acetylation for their localization.

Protein targeting: The ER connection

A simple unicellular organism such as yeast possesses a vast proteome of around 6000 encoded gene products. These proteins are synthesized in the cytoplasm and transported to various subcellular locations. The efficiency of sorting such a vast array of proteins to its various locations is simply astounding. Two organelles long associated with trafficking of proteins are the ER and golgi. The role of these organelles in targeting proteins to the vacuole, plasma membrane, or endosomes is well characterized. There are also reports about the role of ER in targeting integral membrane proteins to the NM (Ostlund et al., 1999, Holmer and Worman, 2001) owing to the continuity of the ER membrane with the ONM. Our screen unearthed the importance of a novel gene *ICE2* in targeting peripheral proteins to the INM. Ice2, an integral membrane protein, localizes to the ER and has been implicated to play a role in maintenance of cortical ER structure and inheritance of cortical ER (de Martin et al., 2005). The exclusivity of Ice2 to the fungi family indicates importance of its role in a pathway unique to fungi such as closed mitosis. Further, the conservation of putative transmembrane domains of Ice2, across species, point towards the significance of these domains. It is probable that Ice2 interacts with other integral membrane proteins via these transmembrane regions or aids in the

integration of other proteins to the ER membrane. These Ice2 interacting proteins might play a role in maintenance of cortical ER. This would attribute an indirect role for Ice2 in maintenance of cortical ER structure and thereby offer an explanation for the defect in its structure in *ice2* Δ mutants (de Martin *et al.*, 2005).

The ER is comprised of tubular structures that spread around the NM (perinuclear ER) and then extend across the cytoplasm towards the periphery of the cell (cortical ER). The ER tubules are highly dynamic and have been shown to constantly redistribute (Prinz *et al*; 2000). In contrast to higher eukaryotic organisms, microtubules and actin do not play a role in maintenance of cortical ER in yeast (Prinz *et al*; 2000) i.e. once the ER tubules migrate towards the periphery, the cytoskeleton is not required for maintaining the structure. In mammals dynamic movement of ER tubules depends on microtubules whereas in yeast, this movement depends on actin. *ice2A* with *kar3A* are synthetically lethal. *KAR3* encodes a microtubule depolymerizing protein and the data indicate that there is association of Ice2, albeit an indirect one, with the functioning of the cellular cytoskeleton. Deciphering the finer details of the interaction between Ice2 and Kar3 would lead to a better understanding of the cellular functions of Ice2.

Chapter 5 illustrates my attempts to decipher the role of Ice2 in targeting a peripheral protein, Trm1-II to the INM. We identified phenotypic similarities between $kar3\Delta$ and $ice2\Delta$ such as elongated microtubules and discovered the lack of a role for microtubules in targeting Trm1-II to INM. We could not discern why $ice2\Delta$ causes Trm1-II to mislocalize to the nucleoplasm. It is highly plausible that Ice2 acts in an indirect manner to direct Trm1-II to its location within the cell. The ER plays an important role in

proteins targeting, defect in the formation of cortical ER in *ice2* Δ may translate to a defect in the targeting of proteins to the NM. These may include integral membrane proteins that functions as the nuclear tether of Trm1-II. In the absence of its tether, Trm1-II will mislocalize to the nucleoplasm. In order to understand the role of Ice2 in mislocalizing Trm1-II, we need a better appreciation for the physiological functions of Ice2. We have initiated studies to observe the effect of *ice2* Δ on the localization of other peripheral INM proteins with localization pattern similar to Trm1-II. These include, Mad1, Mlp2, Esc1 and so on (Huh *et al*; 2003). Our crosslinking initiatives to identify nuclear tether of Trm1-II would also aid in a better understanding of this targeting process.

Peripheral INM proteins: link to diseases

DNA constantly undergoes mutations. Often these do not exhibit an effect on protein function. Nevertheless, some mutations may cause an amino acid change in the catalytic region, or the region of the protein interacting with other cellular components, and this could prove to be lethal for cell viability or cause detectable defects in the cell. In higher organisms, such as humans, these mutations are translated to diseases. Many human diseases are caused due to mutations in DNA that affect particular functions important for the cell. Often, these mutations do not affect the proteins involved in performing the function of interest; rather, they may play an indirect role, leading to mislocalization of the protein, disrupting the interaction of the protein with another in a complex and so on. The nucleus is arguably the most important organelle in the cell and any mutation that disrupts the normal functioning of the nuclear components can lead to development of diseases. One such group of diseases affect the structure, maintenance and functioning of the nuclear envelope and is called laminopathies. These are characterized by mutations in genes encoding the Lamins (Lamin A, B or C and alternate splicing products) as well as various proteins targeted to the nuclear membrane. Many of these mutations, affect interaction of a particular protein with other proteins, and thereby result in a defect in localization or activity of the nuclear envelope component. Emery-Dreifuss muscular dystrophy (EDMD) is caused due to mutation in the gene encoding emerin. Emerin, a protein localized at the INM, mislocalizes in individuals with EDMD (Maraldi *et al.*, 2005). Similarly, the diseases Dunnigan familial partial lipodystrophy (FPLD), limb girdle muscular dystrophy (LGMD), dilated cardiomyopathy (DCM) appear to cause mislocalization of Lamin A and subsequent defects in NM stability (Capanni *et al.*, 2003).

Thus, targeting of proteins to the INM is an important process, failing of which, could lead to disastrous consequences. The role of NLS's in transporting proteins to nucleus is well characterized. What is not clear is the targeting of proteins to sub-nuclear locations such as chromatin, nucleolus, speckles, INM, cajal bodies and so on. The studies described in this thesis uncovered a novel mode of targeting proteins to the INM, by N-terminal acetylation. We have shown this modification to be essential for INM location of Trm1-II-GFP. This modification is highly prevalent in the mammalian genome. It occurs widely in post-translationally modified proteins such as the hormones,

 α -melanocyte-stimulating hormone and β -endorphin. In both cases this modification is important for cellular activity of the hormones. N-terminal acetylation on nascent proteins occurs co-translationally and the function of this modification in higher eukaryotes is largely unknown. Deficiency of this modification leads to various developmental defects in mice, which may be the result of aberrant localization of the proteins essential for developmental processes.

The diverse functions of N-terminal acetylation are coming to light with the discovery of many N-acetylated substrates. In most cases, this modification is essential for the interaction of the modified the protein with another protein, such Arl3 with Sys1, Orc1, Sir3, and Trm1. These interactions may aid in protein sorting, formation of a complex involved in cell cycle control, DNA repair, silencing and various other cellular processes. Studies carried out in yeast could be translated to understand its function in higher organisms and may lead to understanding the defects observed in various human diseases.

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